THE ROLE OF THE CELL-MEDIATED IMMUNE RESPONSE TO ROTAVIRUS INFECTION

by

RICHARD RHEAD HEATH, B.Sc. (Manchester)

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To my mother and father

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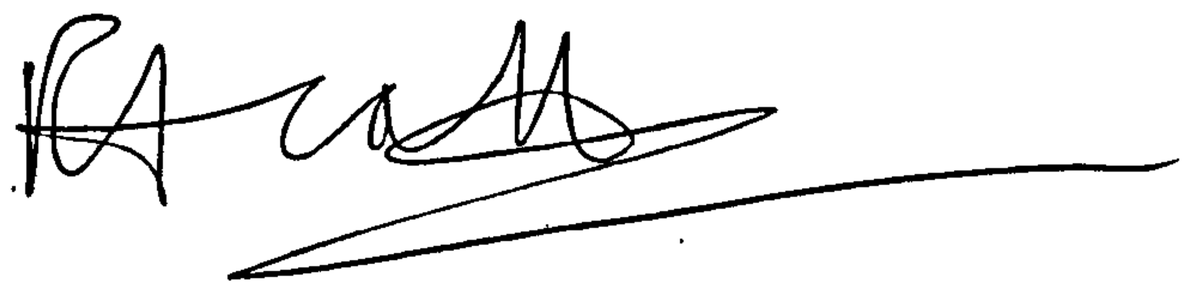
Finally I wish to thank my family and close friends for their love, support and patience over the years which has made this study possible.

DECLARATION

I declare that all the work in this thesis was performed by myself, except the immunofluorescence which was performed by Prof. M. McCrae and N. Broughton and all other work where acknowledged. I also declare, to the best of my knowledge, that this work has not been previously been submitted for a degree in this or any other university.



Richard Heath

SUMMARY

The objective of this project was to determine the protein specificity of the cytotoxic T-lymphocyte (CTL) response to rotavirus infection in mice and to assess the rotavirus serotype/strain independent nature of this response. Previous work, involving the rotavirus group at Warwick, had shown that the outer shell glycoprotein VP7 is a major target antigen for a CTL response that is virus serotype-independent. However, that work did not cover all twelve rotavirus proteins, was confined to one strain of adult mice (C57BL/6, H-2^b) and covered only two of the fourteen VP7 serotypes (serotypes 3 and 6) (Offit *et al.*, 1994).

Recombinant vaccinia viruses expressing individual rotavirus UKtc proteins VP2, VP3, NS26 and NS12 were constructed to complete a set of recombinant vaccinia viruses covering the full complement of rotavirus proteins from the bovine UKtc strain. These were used to define rotavirus proteins eliciting a CTL response in three different mouse haplotypes. UKtc NS53 and UKtc VP7 stimulated a strong CTL response only in the H-2^b MHC class I haplotype (UKtc NS53 and UKtc VP7 were restricted at H-$2D^b$ and H-$2K^b$, respectively). Conversely, UKtc VP3 stimulated a strong CTL response in the H-2^d and H-2^k (but not in H-2^b) MHC class I haplotypes. Work using congenic mouse strains was used to verify that the VP7 protein specific CTL response is restricted solely by the MHC class I antigens.

Rotavirus RRV NS53 was not only found to elicit a CTL response in the H-2^b MHC class I haplotype, similar to UKtc NS53, but also in the H-2^d MHC class I haplotype. Thus, the individual rotavirus protein that elicits a CTL response not only depends on the MHC class I haplotype, but also on the actual rotavirus strain being tested. Many of the previous studies looking at the CTL response to individual rotavirus proteins have, unlike this study, used several different rotavirus strains and, therefore, may have given an inaccurate representation of the rotavirus proteins that elicit a CTL response.

Recombinant vaccinia viruses were also used to examine the serotype/strain independent nature of the CTL response against the major target antigens. The analysis was extended to cover VP3 from two different strains, NS53 from three different strains and VP7 from seven of the fourteen serotypes. VP3 and NS53 were found to elicit a strain-dependent response whereas the serotype-independent nature of the CTL response to VP7 was confirmed. Since the serotype-independent nature of the rotavirus VP7-specific CTL response was found to cross-protect between half of the VP7 serotypes, irrespective of the immunising serotype, it would be reasonable to speculate that the CTL response is serotype-independent between all the VP7 serotypes.

Finally, recombinant vaccinia viruses were used to locate CTL epitopes on NS53 and VP7. Recombinant vaccinia virus expressing a UKtc NS53 deletant mutant (P9DΔ5) showed there to be at least one strain specific epitope in the first 150 amino acids of the UKtc NS53 protein. Recombinant vaccinia viruses expressing four different UKtc VP7 fragments spanning 46% of this protein were examined. It was found that the fragment spanning the restriction enzyme sites at nucleotide 90 (ClaI) and nucleotide 196 (HhaI), i.e. between amino acids 13 and 48 of the mature UKtc VP7 protein, contained a serotype-dependent CTL epitope. The finding suggests that the immunodominant epitope identified in the same region of VP7 by Franco *et al.* (1993) was not the serotype-independent epitope.

LIST OF ABBREVIATIONS

Å	Angstroms
ADCC	Antibody-dependent cell-mediated cytotoxicity
AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
ARC	AIDS-related complex
ATCC®	American Type Culture Collection
ATP	Adenosine triphosphate
B-lymphocyte	Bursa derived lymphocyte
β_2m	β_2-microglobulin
BiP	Immunoglobulin binding protein
bp	Base pairs
BrdU	5'-bromodeoxyuridine
CD	Clusters of differentiation
cDNA	Copy DNA
CIP	Calf intestinal phosphatase buffer
cm	Centimetre
CMV	Cytomegalovirus
cpe	Cytopathic effect
CTL	Cytotoxic T-lymphocyte
CTLp	Precursor CTL
DMEM	Dulbecco's Modification of Eagle's Minimal Essential Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate

ds	Double stranded
DTH	Delayed type hypersensitivity
EBV	Epstein Barr virus
EDTA	Ethylenediamine tetracetic acid
ER	Endoplasmic reticulum
FasL	Fas ligand
FCS	Foetal calf serum
g	Grams
G	Glycoprotein
GMEM	Glasgow Modification of Eagle's Minimal Essential Medium
GTP	Guanosine triphosphate
HA	Hemagglutinin
HBV	Hepatitis B virus
HBsAg	HBV small surface antigen
HCV	Hepatitis C virus
HIV-1	Human immunodeficiency virus 1
HLA	Human leukocyte antigen
hr	Hour
ICAM	Intercellular adhesion molecule
IE	Immediate early
IFN	Interferon
Ig	Immunoglobulin
iIELs	Intestinal intraepithelial lymphocytes
IL	Interleukin
ITAMs	Immunoreceptor tyrosine activation motifs
kDa	Kilodalton

LCMV	Lymphocytic choriomeningitis virus
LFA	Lymphocyte functional antigen
LMP	Low molecular weight polypeptides
m	Messenger
M	Molar
MCMV	Murine cytomegalovirus
MHC	Major histocompatibility complex
μg	Microgram
μl	Microlitre
ml	Millilitre
mM	Millimolar
moi	Multiplicity of infection
MTOC	Microtubule organising centre
NA	Neuraminidase
NEAA	Non-essential amino acids
NK	Natural killer
NS	Non-structural
ORF	Open reading frame
P	Protease sensitive
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pfu	Plaque forming unit
p.i.	Post infection
PKC	Protein kinase C
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase

RER	Rough endoplasmic reticulum
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute 1640 Medium
RPMI DM	Dutch Modified RPMI
RSV	Respiratory syncitial virus
RT-PCR	Reverse transcription polymerase chain reaction
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulphate
SFV	Semliki forest virus
ss	Single stranded
T-lymphocyte	Thymus educated lymphocyte
TAP	Transporter associated with the antigen processing
TCR	T-cell receptor
Th-lymphocyte	Helper T-lymphocyte
TK	Thymidine kinase
TNF	Tumour necrosis factor
ts	Temperature sensitive
U	Units
UV	Ultra violet
VCAM	Vascular cell adhesion molecule
VLA	Very late antigen
VLP	Virus like particle
VP	Viral protein
VR	Variable region
VSV	Vesicular stomatitis virus

WHO	World Health Organisation
WT	Wild-type
Xg	Times gravitational acceleration

CHAPTER 1

THE ROTAVIRUSES

1.1 INTRODUCTION

Rotaviruses were first identified by electron microscopy in 1973 (Bishop *et al.*, 1973; Flewett *et al.*, 1973) and a wealth of knowledge concerning these viruses has been obtained in the last twenty years. They are nonenveloped, icosahedral viruses which belong to the family *Reoviridae* and have a segmented, double-stranded (ds) RNA genome (Kapikian and Chanock, 1990).

Group A rotaviruses are the major etiological agents of acute viral gastro-enteritis, responsible for very large mortality and morbidity problems in humans and economic losses in agriculture. The virus causes a severe dehydrating diarrhoea primarily in the young of both humans, and a wide range of other mammals and birds. In Asia, Africa and Latin America, approximately 850,000 infants die of rotavirus-induced gastro-enteritis each year (WHO, 1989). In developed countries, rotavirus diarrhoea has a low mortality rate but a high morbidity rate as a result of the effective replacement of fluids and electrolytes lost during this viral induced disease. For example, rotavirus gastro-enteritis is believed to cause approximately 517 deaths and 209,000 hospitalisations per year in the United States (Ho *et al.*, 1988; Blacklow and Greenberg, 1991).

1.2 ROTAVIRUS PATHOLOGY AND PATHOPHYSIOLOGY

1.2.1 Intestinal changes following rotavirus infection

Rotavirus infection is often asymptomatic (Zheng *et al.*, 1989). Symptomatic virus infection has an incubation period of up to twenty four hours in the mouse and the infection spreads from the proximal small bowel to the ileum over the following one to two days (Starkey *et al.*, 1986). Although infection normally lasts a total of one week in most animals and humans, the immuno-compromised host can develop a chronic infection which persists for weeks/months or until death (Dharakul *et al.*, 1990; Gilger *et al.*, 1992).

A detailed account of the pathological changes involved in rotavirus diarrhoea is beyond the scope of this thesis and has been recently reviewed by Greenberg *et al.* (1994). Briefly, rotavirus infection is restricted to the mature enterocytes on the tips of the small intestinal villi and leads to mild shortening of the villi, crypt hyperplasia and moderate round-cell infiltration of the lamina propria (Pearson *et al.*, 1978; Pearson and McNulty, 1979). The mechanism of rotaviral induced diarrhoea in the piglet involves a loss of viable absorptive cells which causes an increased faecal lactose loss and an increased faecal osmotic gap (Graham *et al.*, 1984). It has been suggested that the malabsorption of carbohydrates results in an osmotic diarrhoea. The mouse model does not show carbohydrate malabsorption (Collins *et al.*, 1988) and in this case diarrhoea is thought to arise from villus ischemia leading to a secondary net intestinal secretion (Osborne *et al.*, 1988). Very recent work by Ball *et al.* (1996) showed that the non-structural rotavirus protein NS28 is a viral enterotoxin in the mouse. They speculated that NS28 is released into the lumen from infected cells and then binds to a receptor on adjacent cells thus triggering intestinal secretory pathways.

1.2.2 Age restriction of rotavirus infection

Rotavirus associated diarrhoea typically occurs in 21% of children less than 6 months old, 68% of children between 6-24 months old and only 8% of children between 24 months to 13 years old (Kapikian *et al.*, 1976). This apparent age restriction relates only to disease and not to asymptomatic rotavirus infection (Bridger, 1994).

Proteolytic cleavage of rotaviral protein VP4 into VP5* and VP8* with trypsin is thought to be required for the efficient entry of rotavirus into tissue culture cells (Suzuki *et al.*, 1985; Kaljot *et al.*, 1988). Bass *et al.* (1992) found that the adult mouse stomach cleaved VP4 in a manner distinct from that of the suckling stomach (which cleaved VP4 similarly to trypsin) and that rotaviruses showed a 10,000-fold decrease in titre following

passage through the adult stomach when compared to the passage through the suckling stomach. It was suggested that the development of gastric acid and pepsin secretions may offer the protection seen with increased age.

The age dependency of rotavirus disease could also be conditional on the quantity of rotavirus-specific binding receptors on the epithelial cells (Riepenhoff-Talty *et al.*, 1982) and/or the immunity gained following the asymptomatic infection of neonates with the nursery strains of rotavirus (Albert *et al.*, 1987).

1.3 STRUCTURE OF THE ROTAVIRUS PARTICLE

Cryo-electron microscopy and computer image processing allowed the three dimensional structure of the virus particle to initially be determined to a resolution of 40 angstroms (Å) (Prasad *et al.*, 1988) and more recently to a resolution of 26Å as shown in Figure 1 (Yeager *et al.* 1994).

The spherical rotavirus particle (377Å in mean diameter) has a T=131 icosahedral surface lattice. In the centres of the 120 six-co-ordinated and 12 five-co-ordinated points are aqueous channels, 132 in all, which are approximately 55Å in diameter and 140Å in depth. These channels have been suggested as having a role in the transit of metabolites and nascent RNA transcripts in and out of the particle, respectively (Prasad *et al.*, 1988).

The triple-shelled virus particle is composed of the outer shell proteins VP4 and VP7. The 780 molecules of the VP7 protein form a smooth protein surface and the VP4 dimers (200Å in length and in contact with VP6 and VP7) form 60 spikes which are located next to the channels surrounding the icosahedral five-fold axis. Buried within the virion, the VP4 dimer NH_2-terminus has a hexagonal base (85Å in diameter). The VP4 spike protrudes 110Å from the surface of the virion and has a globular domain 55Å in diameter (Prasad *et al.*, 1990; Shaw *et al.*, 1993).

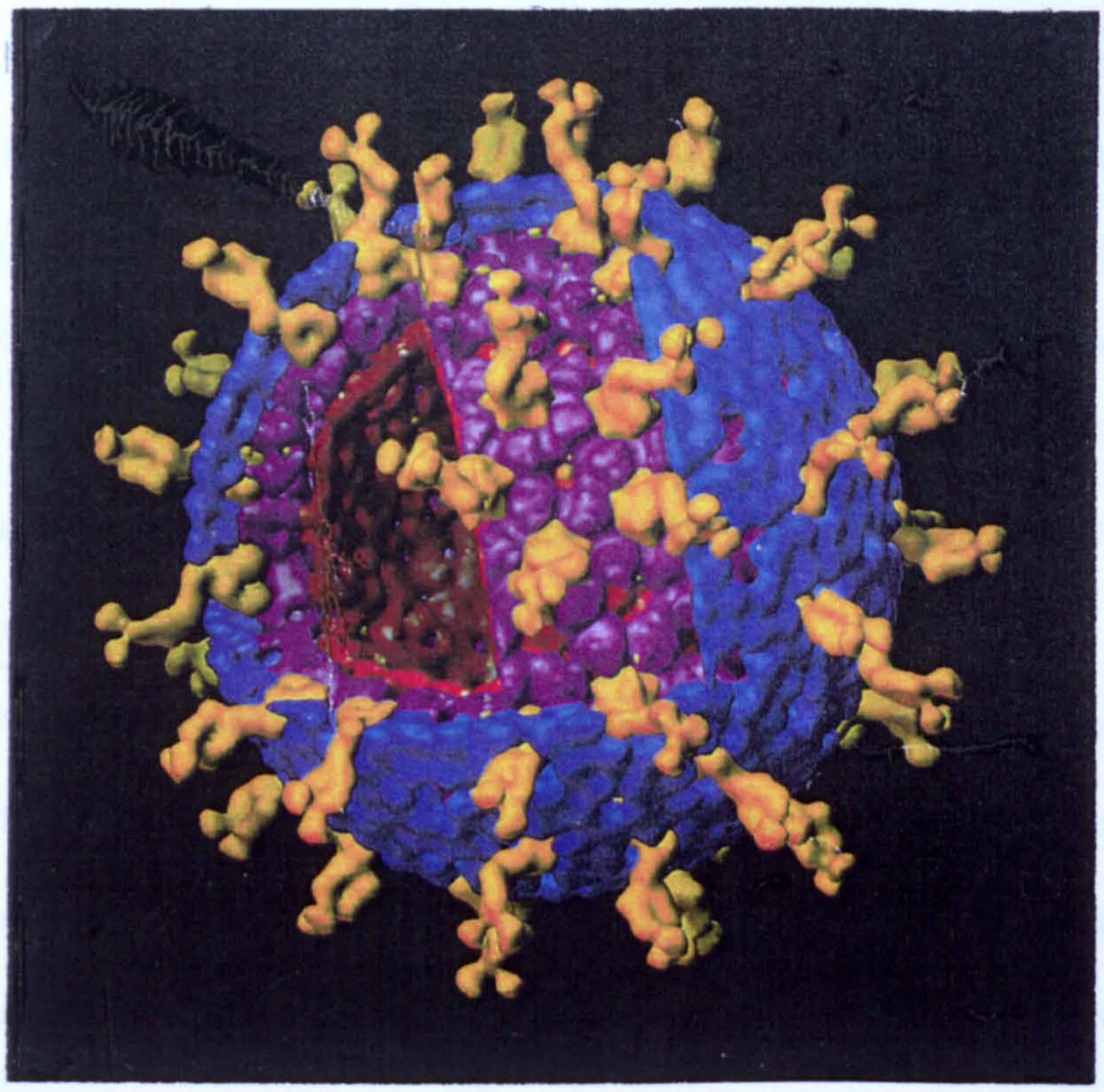


FIGURE 1. COMPOSITE RECONSTRUCTION OF THE ROTAVIRUS SA11 PARTICLE

A computer generated image of a rotavirus SA11 particle prepared using cryo-electron microscopy at a resolution of 26Å (Yeager *et al.*, 1994). A segment of the particle has been removed to allow visualisation of the three concentric protein shells which form the mature virus. The outer shell proteins VP4 (yellow) and VP7 (blue), the intermediate shell protein VP6 (purple) and the inner shell protein VP2 (red) are distinguishable. VP1 and VP3 are minor components of the virion core and are associated with the 11 segments of double stranded RNA (not shown). A total of 132 aqueous channels extend through the outer (VP7) and intermediate (VP6) shells to the virion core.

The outer shell of rotavirus (VP4 and VP7) can be removed using chelating agents such as ethylenediamine tetracetic acid (EDTA) to reveal what has historically been referred to as the single-shelled virus particle, although, the cryo-electron microscopy studies indicated that it is infact a double-shelled structure. The surface of this particle (299Å in mean diameter) is composed of 260 trimers of VP6 and follows the same T=131 icosahedral symmetry as the outer surface. The arrangement of the VP6 trimers form channels that run through this layer in accordance with the channels observed in the outer shell (Yeager *et al.*, 1990).

Beneath VP6 there is the single-shelled 'core' (255Å in mean diameter) composed of 180 molecules of the VP2 protein which follows the same T=131 icosahedral symmetry as VP6 (Yeager *et al.*, 1990). In fact, empty VP2 particles, formed in insect cells infected with recombinant baculovirus containing rotavirus gene 2, showed that this layer could be made up of three VP2-related proteins [of molecular weights 94, 85 and 77 kilodaltons (kDa)] due to expression of truncated forms of VP2 (Zeng *et al.*, 1994).

Beneath the VP2 layer, there is speculation that a further structural entity exists 'the subcore' which is composed of the VP1 and VP3 minor proteins and the genome RNA (Prasad and Chui, 1994).

1.4 THE ROTAVIRUS GENOME

The rotavirus genome consists of 11 dsRNA segments which can be separated by polyacrylamide gel electrophoresis and the total average size of the genome is 18,550 base pairs (bp) (Desselberger and McCrae, 1994). The genome segments have conserved terminal sequences (5'....GGC$^{A}/_{U}{}^{A}/_{U}U^{A}/_{U}A^{A}/_{U}{}^{A}/_{U}$....3' and 5'....$^{A}/_{U}U^{G}/_{U}{}^{U}/_{G}{}^{G}/_{U}{}^{A}/_{G}$CC....3' for the 5' and 3' ends, respectively) (Imai *et al.*, 1983; McCrae and McCorquodale, 1983) and short non-coding sequences (up to 50 bp and 185 bp for the 5' and 3' ends, respectively) (Desselberger and McCrae, 1994). The conserved 5' end of all the genes

contains a cap (5' m^7GpppG(m)GC....3') and all the genes are A+U rich (58 to 67%). The genes, however, have no 3'-terminal poly(A) tails (Imai *et al.*, 1983; McCrae and McCorquodale, 1983). The untranslated 5' and 3' primary sequences and secondary structures may serve as recognition signals for the RNA binding proteins involved in RNA expression, synthesis and packaging (Clarke and McCrae, 1982). With one exception (gene 11), the genes have one reading frame and, in most cases, use the first initiation codon.

1.5 PROPERTIES OF ROTAVIRUS PROTEINS

1.5.1 Rotavirus protein nomenclature

The nomenclature of rotavirus proteins changes frequently and in this thesis the proteins will be named as viral proteins (VP) followed by a number of the structural protein, and non-structural (NS) proteins followed by a number indicating the molecular weight on gels (in thousands) (Mason *et al.*, 1983; Liu *et al.*, 1988). Where possible the order of proteins in this thesis relates directly to the genome pattern (i.e. gene 1 (VP1), 3302 bp through to gene 11 (NS26), 667 bp), however, it should be noted that RNA segments 7 to 9 (and also 10 and 11) have differing migration patterns depending on the rotavirus strain. The following gene-protein assignment is that for the bovine rotavirus strain UKtc.

1.5.2 Gene segment 1 (VP1)

Gene 1 (3302 bp) encodes the VP1 structural protein (125 kDa) which is a minor component of the virion core and comprises 2% of the virion mass (Liu *et al.*, 1988). The protein shows a high level of homology between different strains, 96% at the protein level (Both *et al.*, 1994).

There is evidence to suggest that VP1 is the viral RNA-dependent RNA polymerase in that it has conserved sequence homologies (between amino acids 517 and

636) to genes encoding putative RNA polymerases of other viruses (Cohen *et al.*, 1989). In addition, VP1 becomes cross-linked by the nucleotide analogue [α-^{32}P] azide-ATP in the presence of ultra-violet (UV) light and viral mRNA synthesis was decreased (Valenzuela *et al.*, 1991).

1.5.3 Gene segment 2 (VP2)

Gene 2 (2691 bp) encodes the VP2 structural protein (94 kDa) which is the major component of the single-shelled virion core and comprises 12 to 15% of the virion mass (Liu *et al.*, 1988). The protein shows a high level of homology between different strains, 91% at the protein level (Both *et al.*, 1994), and is accessible to VP2-specific antibodies even as a component of the double-shelled particle (Taniguchi *et al.*, 1986).

The conserved amino acid residues between 535 to 556 and 666 to 687 of the NH_2-terminal half of VP2 contain leucines every seventh position which are hypothesised to form a 'leucine zipper', a structure believed to be involved in the dimerisation of nucleic acid binding proteins (Kumar *et al.*, 1989). Temperature sensitive (ts) mutants suggest the protein region around amino acid 387 may be involved in core assembly (Mansell *et al.*, 1994).

VP2 has been suggested to exhibit dsRNA replicase activity since VP2 core-like particles are the simplest structures that can bind and synthesise dsRNA (Mansell and Patton, 1990; Labbe *et al.*, 1991). The use of baculovirus expressing truncated VP2 proteins by Labbe *et al.* (1994) localised the non-specific nucleic acid binding domain between amino acids 1 to 132 and showed higher affinity binding of ss- than dsRNA and DNA. These experiments also revealed UV cross-linking of genomic RNA to only the VP2 protein in double-shelled particles and it was speculated that VP2 may package viral mRNA into the core during replication.

1.5.4 Gene segment 3 (VP3)

Gene 3 (2591 bp) encodes the VP3 structural protein (88 kDa) which is a minor component of the virion core and comprises 0.5% of the virion mass (Liu *et al.*, 1988).

· VP3 is thought to be the viral guanylyltransferase responsible for RNA capping due to the specific and reversible binding of GTP (Pizarro *et al.*, 1991; Liu *et al.*, 1992).

1.5.5 Gene segment 4 (VP4)

Gene 4 (2362 bp) encodes the VP4 structural protein (88 kDa) which is a minor component of the outer capsid and comprises 1.5% of the virion mass (McCrae and McCorquodale, 1982; Liu, *et al.*, 1988).

Dimers of VP4, as described in section 1.3, form the 60 spike like structures on the surface of the virion. Proteolytic cleavage of VP4 into VP5* (60 kDa) and VP8* (28 kDa) occurs at amino acid sites arginine (Arg)-241 and Arg-247 releasing six nucleotides (Lopez *et al.*, 1985). Arg-241 and Arg-247 are conserved between virus isolates, however, considerable amino acid variation occurs between, and adjacent to, the cleavage sites (Lopez *et al.*, 1986) and this region of VP4 has been speculated to influence rotavirus virulence.

VP4 has a number of important characteristics being the viral hemagglutinin (Fuetes-Panana *et al.*, 1995) and carrying neutralising antibody epitopes (Hoshino *et al.*, 1985). (For an analysis of VP4 serotypes the reader is referred to section 1.7.3). There is evidence to suggest that VP4 has an important role in the growth and virulence of rotavirus *in vitro* (Burke *et al.*, 1994; Taniguchi *et al.*, 1994), but *in vivo* studies show conflicting results as to its involvement in rotavirus virulence (Flores *et al.*, 1986; Broome *et al.*, 1993).

There is some debate as to the identity of the cell attachment protein of rotavirus (there being evidence in favour of both VP4 and VP7) and the argument for VP4 will be

discussed here. There appeared to be two routes of uptake of rotavirus, endocytosis and direct penetration (Suzuki *et al.*, 1985), however, Kaljot *et al.* (1988) stopped endosomal formation with no decrease in virus yield. This result suggested a limited role for endosomal uptake and complemented the proposed fusion domain sequences found between amino acids 137 and 154 of VP5* (Mackow *et al.*, 1988b). Neutralising antibody epitopes also map to this region of VP5* (Mackow *et al.*, 1988b) although actual virus attachment was blocked by neutralising antibodies to VP8* (and not to VP5*) (Ruggeri and Greenberg, 1991). In addition, baculovirus expressed proteins forming virus-like particles (VLPs) indicated VP4 to be the viral attachment protein (Crawford *et al.*, 1994), a conclusion also reached by Ludert *et al.* (1996) using reassortment viruses containing VP4 of different parental strains (which differ in their requirement for sialic acid during binding).

1.5.6 Gene segment 5 (NS53 or NSP1)

Gene 5 (1581 bp) encodes the NS53 non-structural protein (53 kDa) (Mason *et al.*, 1983). The protein shows variable levels of amino acid conservation (34 to 96%) with the greatest diversity between strains of different species (Xu *et al.*, 1994).

The function of NS53 is still unclear, however, the protein does contain a conserved sequence motif between amino acids 37 and 81 encoding a metal binding domain (Xu *et al.*, 1994). Proteins containing metal binding domains are known to associate with nucleic acids and, indeed, NS53 has been shown bind to all 11 rotavirus mRNAs (Hua *et al.*, 1994). NS53 is present throughout the cytoplasm in association with cytoskeleton and there is evidence to suggest that NS53 binds to the 5' end of the rotavirus mRNA (Hua *et al.*, 1994). It is speculated that NS53 may redirect the mRNA towards encapsulation and particle formation, rather than protein translation.

1.5.7 Gene segment 6 (VP6)

Gene 6 (1356 bp) encodes the VP6 structural protein (41 kDa) which is the most abundant component of the virion, comprising 50% of the virion mass (Liu *et al.*, 1988).

VP6 is the outer protein of the double-shelled virus particle and occurs as a trimer (see section 1.3). VP6 contains both the group-specific antigens and sub-group A-specific antigens as detailed in section 1.7.1. In addition, Chen and Ramig (1993) showed that the transcriptase activity of the double-shelled particle is dependent on the presence of VP6.

The VP6 protein of the double-shelled viral particle binds to the NS28 protein spanning the rough endoplasmic reticulum (RER) membrane prior to the budding of the particle into the lumen of the RER during the assembly process (Au *et al.*, 1989; Meyer *et al.*, 1989; Suzuki *et al.*, 1993). The region of the VP6 protein which binds with NS28 is not known but is speculated to be conserved between rotavirus strains (Meyer *et al.*, 1989).

1.5.8 Gene segment 7 (NS35 or NSP2)

Gene 7 (1059 bp) encodes the NS35 non-structural protein (35 kDa) (McCrae and McCorquodale, 1982; Mason *et al.*, 1983) which is associated with viroplasms in infected cells (Petrie *et al.*, 1984).

NS35 is thought to be essential for genome packaging and replication since viruses carrying a ts mutation in the corresponding gene produce RNA deficient particles at the restrictive temperature (Ramig and Petrie, 1984; Gombold *et al.*, 1985). More recently, NS35 has been shown to have a non-specific RNA binding capability (Kattoura *et al.*, 1992) and to form 10S multimers, probably consisting of 6 to 8 copies of the protein, during infection of the cell (Kattoura *et al.*, 1994).

1.5.9 Gene segment 8 (VP7)

Gene 8 (1062 bp) encodes the VP7 structural glycoprotein (34 kDa) which is the major component of the outer capsid and comprises 30% of the virion mass (Both *et al.*, 1983; Liu *et al.*, 1988). The VP7 protein of almost all rotavirus strains is high mannose glycosylated at amino acid 69 and the protein has three other potential glycosylation sites at amino acids 146, 238 and 318 (Both *et al.*, 1994). VP7 also contains neutralising antibody antigens which are detailed in section 1.7.2.

The gene contains two initiation codons in the same open reading frame (ORF) encoding for proteins 326 and 286 amino acids in length and, although the second initiation codon has a stronger consensus sequence, both proteins are detected in infected cells (Chan *et al.*, 1986). Both VP7 proteins start with hydrophobic signal sequences (H1 and H2) which may direct the nascent VP7 to the RER and both signal sequences appear to be cleaved at amino acid 51 (Stirzaker *et al.*, 1987). The mechanism responsible for retaining the mature VP7 protein in the RER is not known. The H2 signal peptide and the first 31 amino acids of the mature VP7 protein have been shown to be involved in this process (Maass and Atkinson, 1994). However, because the uncleaved VP7 precursor has not yet been identified in cells (Stirzaker and Both, 1989), it has been speculated that retention involves the cleaved H2 signal peptide interacting with the first 31 amino acids of the mature VP7 protein.

As mentioned in section 1.5.5, the identity of the cell attachment protein of rotavirus is debated, there being evidence in favour of both VP4 and VP7. VP7 is believed to be the cell attachment protein because purified VP7 binds to cells and, indeed, binding of infectious virus was inhibited by both monoclonal antibodies directed to VP7 and by competitive inhibition using the purified VP7 protein (Sabara *et al.*, 1985, Fukuhara *et al.*, 1988).

1.5.10 Gene segment 9 (NS34 or NSP3)

Gene 9 (1104 bp) encodes the NS34 non-structural protein (34 kDa) (Mason *et al.*, 1983), however, its function is still unknown.

NS34 was found to be associated with the cytoskeleton of the infected cell (Mattion *et al.* 1992) and to bind to the conserved 3' terminal mRNA sequences in a rotavirus group specific manner (Poncet *et al.*, 1994). Poncet *et al.* (1994) suggested a role for NS34 in the assembly of the 11 viral mRNAs during precore formation.

1.5.11 Gene segment 10 (NS28 or NSP4)

Gene 10 (751 bp) encodes NS20 which is post-translationally glycosylated forming the NS28 non-structural protein (28 kDa) (McCrae and McCorquodale, 1982; Mason *et al.*, 1983).

Three amino-terminal hydrophobic regions (H1 to H3 between amino acids 7 to 21, 28 to 47 and 67 to 85, respectively) anchor the protein to the RER, H2 spanning the membrane, and H1 containing two high mannose glycosylation sites (within RER lumen). The carboxyl-terminal half of NS28 protrudes into the cytoplasm of the cell (Bergmann *et al.*, 1989).

Membrane anchored NS28 is involved in particle maturation and binds double-shelled particles via the VP6 protein with an affinity constant of 4.6 X 10^{-11} M. The tetrameric nature of NS28 and the trimeric nature of VP6 suggests a co-operative interaction between many receptors leading to the relatively high affinity constant (Taylor *et al.*, 1993). A region of the NS28 protein critical for the binding of the double-shelled particle was located between amino acids 161 to 172 and binding was also highly dependent on the conformational integrity of the carboxyl-terminus (Au *et al.*, 1993). NS28 also forms oligomeric structures with VP4 and VP7 during particle budding and assembly (Maass and Atkinson, 1990).

1.5.12 Gene segment 11 (NS26/NS12 or NSP5/NSP6)

Gene 11 (667 bp) contains two initiation codons in different ORFS and encodes two non-structural proteins. The larger protein, NS26 (26 kDa), is phosphorylated and modified by the addition of O-linked N-acetylglucosamine monosaccharide residues which increase the molecular weight of the protein (28 kDa) (Welch *et al.*, 1989; Gonzalez and Burrone, 1991). The smaller protein, NS12 (11 kDa), has also been shown to be expressed in infected cells (Mattion *et al.*, 1991). The functions of the NS26 and NS12 proteins remain unclear.

Gene 11 from different rotavirus strains has been found to exhibit differing migration patterns following RNA gel electrophoresis (long, short and super-short) and this is due to gene rearrangements (see section 1.6.3) (Nuttall *et al.*, 1989).

1.6 ROTAVIRUS GENETIC DIVERSITY

Section 1.5 describes the rotavirus genome consisting of eleven segments of dsRNA encoding twelve individual viral proteins. Sequence analysis of individual genes from rotaviruses isolated world-wide shows that genetic variation exists. The variation is thought to arise from three distinct mechanisms: point mutation, reassortment and rearrangement. Variation in the viral proteins involved in stimulating the hosts immune response may present an additional obstacle in the development of an effective vaccine.

1.6.1 Point mutation

The sequential accumulation of point mutations has not yet been observed in rotavirus isolates obtained from sequential epidemic seasons (Palombo *et al.*, 1993). In theory, however, each rotaviral protein is subjected to varying host immune selection pressures and thus mutation frequencies. Resistant rotavirus mutants generated *in vitro* by culturing

the virus in the presence of monoclonal antibodies have provided an estimated mutation rate for the two outer capsid antigens (VP4 and VP7) of approximately 10^{-5} base substitutions per position per virus generation (Taniguchi and Urasawa, 1995). Host immune selection pressures may also act on the NS53 protein since gene 5 shows a great genetic diversity among strains from different species (Xu *et al.*, 1994). However, being a non-structural protein, an antibody-mediated immune selection pressure would seem unlikely and, in fact, a cell-mediated immune response may provide the selection pressure (S. Stagg, personal communication).

1.6.2 Reassortment

Coinfection of mice with two different rotavirus strains resulted in 80 to 100% of the progeny virus containing assortments of gene segments by 96 hours post infection, however, gene segments 3 and 5 were found to segregate at a relatively low frequency (Gombold and Ramig, 1986). The co-segregation of certain genes was also found between gene 6 and gene 11 (Kobayashi *et al.*, 1994) and the implication is that the rotavirus genome is adapted for optimal function as a group.

It is not known how the assortment and packaging of viral RNA segments is regulated, although, there is speculation that the 5' and 3' untranslated regions may serve as recognition signals for the RNA binding proteins involved in this process. However, sequence diversity in the 5' and 3' untranslated regions is minimal and, consequently, the co-segregation of certain genes during rotavirus coinfection cannot be explained by sequence divergence in these regions (Clarke and McCrae, 1982). Interestingly, the host can also affect the reassortment frequency of specific genotypes which was discovered by comparing reassortment experiments in different cell lines (Graham *et al.*, 1987).

The phenotype of a reassortant virus is unpredictable because the background proteins of the recipient can influence the structure of the donor protein (Chen *et al.*, 1989;

Dunn *et al.*, 1994). For example, Chen *et al.* (1992) found using reassortants that the presence of a neutralising epitope on VP4 depended on the VP7 background. Therefore, reassortment could, in theory, lead directly to new serotypes since VP4 and VP7 appear to influence the conformation of each other and their antigenic sites.

Ramig (1990) showed that a second viral infection of a cell line, twenty-four hours after the first, still resulted in the formation of reassortants. This absence of superinfection exclusion increases the potential for reassortment in the natural environment. In addition, natural rotavirus coinfections appear to be more frequent in developing countries than in more developed countries. For example, from 1986 to 1992, nineteen percent of patients showed coinfections in Brazil compared with just 1% of patients in Hungary (Timenetsky *et al.*, 1994; Szucs *et al.*, 1995). This may be due to lower levels of hygiene and frequent overcrowding in the developing countries. Although there is a potential in the natural environment for the appearance of new rotavirus strains by reassortment (Matsuno *et al.*, 1988; Shif *et al.*, 1994), the emergence and rapid spread of highly virulent rotavirus strains (as seen with the pandemic strains of influenza virus) has yet to be observed.

1.6.3 Rearrangement

Various rotavirus genes (5, 6, 8, 10 and predominantly 11) can undergo rearrangements which, after polyacrylamide gel electrophoresis, are characterised by the RNA segment being missing from its normal position or by the appearance of additional dsRNA bands. This phenomenon has been detected in the natural infections of many animal species (Mattion *et al.*, 1988; Tanaka *et al.*, 1988; Scott *et al.*, 1989), immunocompromised humans (Pedley *et al.*, 1984; Hundley *et al.*, 1987) and following the serial high passage of rotavirus in tissue culture cells (Hundley *et al.*, 1985).

Examples of gene rearrangements include the UKtc variant P9DΔ5 which has a shorter genome segment 5 due to a 308 base pair deletion starting at 460 bp which leads to a frame shift and to the generation of a premature stop codon a further 24 bp downstream of the deletion. A truncated form of NS53 consisting of the first 150 amino acids is encoded (Tian *et al.*, 1993). The enlarged genome segment 10 of a rotavirus isolated from an immunodeficient child showed a partial duplication of the open reading frame (ORF) inserted immediately after the termination codon (Ballard *et al.*, 1992). It has been speculated that this type of change in the viral genome is generated by the virus-associated RNA-dependent RNA polymerase falling back on its template during replication.

The genes encoding the outer capsid proteins, which independently stimulate neutralising antibodies, have not been found to undergo rearrangement and, in fact, all affected genes found to-date have encoded non-structural proteins. However, since it appears that non-structural proteins (specifically NS53) stimulate cell-mediated immune responses (S. Stagg, personal communication) and viruses with genome rearrangements are not defective, this mechanism of variation has the potential to influence the antigenicity of the virus. It should be noted, however, that many of the rearrangements do not affect the coding potential of the genes concerned.

1.7 ROTAVIRUS GROUPS, SUBGROUPS AND SEROTYPES

1.7.1 VP6 contains the group and subgroup specific antigens

VP6 is the major protein of the rotavirus particle, it is highly immunogenic but results in the production of non-neutralising antibodies (Svensson *et al.*, 1987a/b). Initial serological studies identified that rotaviruses from diverse species shared common antigens which resided on VP6 (named 'conventional' viruses) (Woode *et al.*, 1976). More recently, rotaviruses lacking the common group antigen were identified and, consequently, the

viruses were separated into groups, A (the conventional viruses) to E, depending on their distinctive common antigen (Pedley *et al.*, 1983/1986).

The group A rotaviruses are a common cause of enteric disease in the young of most species. However, although non-group A rotaviruses have been identified in both humans and many animal species, the prevalence and significance of non-group A rotavirus infections still remains unclear. A description of the non-group A rotaviruses is beyond the scope of this thesis but a detailed review has recently been published by Saif and Jiang (1994).

Subgroup determinants are also located on VP6 which divide group A rotaviruses into subgroup I (most animal and certain human rotavirus strains), subgroup II (most human and a few porcine and lapine rotavirus strains), subgroup I and II (rare human or animal rotavirus strains) and neither subgroup I or II (rare human and animal strains and most avian rotavirus strains) (Kapikian *et al.*, 1981; Hoshino *et al.*, 1987) (see Table 1).

VP6 group specificity is thought to be determined by continuous determinants (Gorziglia *et al.*, 1988). Sequence analysis has revealed four conserved VP6 regions (between amino acids 66 to 79, 102 to 113, 133 to 144 and 352 to 374) which may contain group epitopes (Ito *et al.*, 1995). Studies using VP6 monoclonal antibodies against a panel of consecutive VP6 heptapeptides also identified four antigenic regions on VP6, but, in different locations from the aforementioned study (amino acids 32 to 64, 155 to 167, 208 to 274 and 380 to 397) (Kohli *et al.*, 1993).

The subgroup specificity is thought to be determined by conformational epitopes which are present on the trimeric, but not the monomeric, VP6 (Gorziglia *et al.*, 1988; Liprandi *et al.*, 1990). Regions of VP6 that have been speculated to be part of the subgroup specific antigenic site(s) include: twelve conserved amino acids grouped into five regions (Gorziglia *et al.*, 1988), conserved amino acids at positions 120, 317 and 350 (Ito *et al.*,

Table 1. P serotype (VP4 genotype), G serotype and subgroup of human and animal group A rotaviruses. From Hoshino and Kapikian (1994) and Taniguchi and Urasawa (1995).

VP4 genotype	VP4 (P) serotype	Rotavirus Strain	VP7 (G) serotype	VP6 Subgroup	Host species
1	P6	NCDV, J2538, C486	G6	I, I, ?	Calf
	P6	A5	G8	?	Calf
	P6	SA11 (4FEM, 4F, 4fM)	G3	I	Monkey
2	?	SA11 (4SEM, TN-L2, TN-S1)	G3	I	Monkey
3	P5	RRV	G3	I	Monkey
	?	K9, CU-1	G3	I	Dog
	?	Cat97	G3	I	Cat
	?	HCR3	G3	I	Human
4	P1B	DS-1, RV-5, S2	G2	I	Human
	P1B	L26	G12	I	Human
5	P7	Uktc, B641, IND, OK	G6	I	Calf
	P7	61A	G10	I	Calf
6	P2A	M37	G1	II	Human
	P2A	1076	G2	I	Human
	P2A	McN13, RV-3, WCN	G3	II, II, ?	Human
	P2A	ST-3	G4	II	Human
	P2B	Gottfried	G4	II	Pig
7	P9	OSU, TRF-41	G5	I	Pig
	P9	YM, A243.1	G11	I	Pig
	P9	CRW8, Ben-307	G3	I, ?	Pig
	P9	BMI-1, SB-1A	G4	?, II	Pig
8	P1A	KU, Wa	G1	II	Human
	P1A	P, YO, MO	G3	II	Human
	P1A	VA70, Hochi, Hosokawa	G4	II	Human
	P1A	WI-61, F45	G9	II	Human
9	P3A	K8	G1	II	Human
	P3A	AU-1	G3	I	Human
	P3A	PA151	G6	I	Human
	P3B	Mc35	G10	I	Human
	?, P3A	Cat2, FRV-1	G3	I	Cat
10	P4	57M	G4	II	Human
	P4	69M	G8	I	Human
11	P8	B223, A44, KK3, B-11, Cr	G10	I, ?, I, I, I	Calf
	P8	116E	G9	I	Human
	P8	I321	G10	I	Human
12	?	H2, FI-14	G3	not I or II, I+II	Horse
	?	FI23	G14	I	Horse
13	?	MDR13	G3	?	Pig
14	P3B	Mc35	G10	I	Human
15	?	Lp14	G10	?	Sheep
16	P10	Eb	G3	I	Mouse
17	?	993/83	G7	?	Calf
18	P11	PA169	G6	I	Human
	P11	HAL1166	G8	?	Human
19	?	L338	G13	I	Horse

1995) and, with the use of mutants, amino acids at positions 172 and 305 (Lopez *et al.*, 1994).

1.7.2 VP7 contains the G serotype specific antigens

VP7 comprises the smooth layer of the outer shell of the rotavirus virion and contains major neutralising epitopes. To date, fourteen distinct G (for glycoprotein) serotypes have been defined for group A rotaviruses (see Table 1) and nine of the G serotypes have been identified in humans, most commonly G1 to G4 (Beards *et al.*, 1992; Gerna *et al.*, 1992; Woods *et al.*, 1992).

Initial studies mapping the regions of VP7 involved in neutralisation applied the sequencing of the gene encoding VP7 from rotavirus mutants resistant to neutralising monoclonal antibodies (Dyall-Smith *et al.*, 1986; Mackow *et al.*, 1988a). This work suggested that three regions on VP7 determined the binding of serotype-specific neutralising antibodies (between amino acids 87 to 96, 145 to 150 and 211 to 223, termed A, B and C, respectively) and that regions A and C were in close proximity in the native glycoprotein, thus forming a conformational domain. In fact, the conformation of VP7 (conserved cysteine residues forming disulphide bonds) has been shown to be important for functional neutralisation epitopes (Svensson *et al.*, 1994). Further analysis of eight serotypically distinct VP7 amino acid sequences identified nine variable regions (VR1 to VR9) of which VR5, VR7 and VR8 (between amino acids 87 to 101, 142 to 152 and 208 to 221, respectively) corresponded to the regions A, B and C (Green *et al.*, 1989). Subsequently, it was shown that the majority of serotype-dependent neutralisation sites were located in VR5, VR7 and VR8 and, in addition, VR5 also contained the majority of serotype-independent neutralisation sites (Mackow *et al.*, 1988a; Taniguchi *et al.*, 1988a; Coulson and Kirkwood, 1991; Kobayashi *et al.*, 1991a/b).

1.7.3 VP4 contains the P serotype specific antigens

The VP4 protein forms the spikes which protrude from the outer shell, is the viral hemagglutinin and, like VP7, contains major neutralising epitopes. The VP4 and VP7 neutralising antigens segregate independently of each other (Offit *et al.*, 1986a). Eleven VP4 P (for protease) serotypes have been distinguished to-date and serotypes 1 to 3 are further distinguished into subtypes A and B depending on critical amino acid substitutions (see Table 1) (Gorziglia *et al.*, 1990a). However, the P serotypes are difficult to differentiate serologically and, consequently, comparative VP4 amino acid homology sequence analysis (named 'the VP4 genotype') is used to aid differentiation (Estes and Cohen, 1989). To-date, nineteen distinct VP4 genotypes have been defined for group A rotaviruses and nine of the VP4 genotypes have been identified in humans. Genotypes 4, 8 to 10 and 14 have been found to be most commonly associated with symptomatic infections (see Table 1) (Qian and Green, 1991; Das *et al.*, 1993).

Initial studies mapping the regions of VP4 involved in neutralisation applied the sequencing of gene 4 from rotavirus mutants resistant to neutralising monoclonal antibodies (Mackow *et al.*, 1988b; Taniguchi *et al.*, 1988b; Gorziglia *et al.*, 1990b; Kobayashi *et al.*, 1991c). This work identified that most VP4 amino acid substitutions generated by serotype-dependent antibodies mapped to the large hypervariable region in the VP8* subunit (between amino acids 92 and 192), whereas, amino acid substitutions generated by cross-neutralising antibody mapped to the conserved VP5* subunit (between amino acids 388 and 441). The binding of neutralising antibodies to synthetic peptides (18 to 21 amino acids in length), corresponding to the regions around the position where each mutant had sustained its amino acid substitutions, suggested that some of these regions contained conformational epitopes and others contained linear epitopes (Taniguchi *et al.*, 1988b).

1.8 EPIDEMIOLOGY OF ROTAVIRUS INFECTION

Rotaviruses are transmitted by the faecal-oral route (Ward *et al.*, 1986). The world-wide distribution of the virus leads to the universal acquisition of rotavirus-specific serum antibody in 90% of all populations by the end of the third year of life and high rotavirus-specific serum antibody levels are maintained into adult life (Echeverria *et al.*, 1983; Brussow *et al.*, 1988).

As mentioned in section 1.7, nine VP7 (G) serotypes have been found in humans, G1 to G4 being most common and, in addition, nine VP4 genotypes have been detected in humans, VP4 genotypes 4, 8 to 10 and 14 being frequently associated with disease. The VP4 and VP7 proteins can segregate independently of each other (Offit *et al.*, 1986a), however, there is a correlation between the G serotype and the VP4 genotype of the circulating viruses (Timenetsky *et al.*, 1994). Several G serotypes can co-circulate in a community (Gomez *et al.*, 1990; Padilla-Noreiga *et al.*, 1990) and initial studies suggest that a similar co-circulation of VP4 genotypes exists (Das *et al.*, 1994; Timenetsky *et al.*, 1994).

In general, the epidemiological patterns of rotaviruses are very complex and the predominating serotype circulating within a given population appears to change over time. To-date, no clear patterns of change which would permit predictions of the predominant serotypes have been discovered. In addition, the genetic processes driving the antigenic change are not known, however, mechanisms such as sequential point mutations in the antigenic regions of the genes encoding VP4 and VP7 (see section 1.6.1) and reassortment of the rotaviral genome (see section 1.6.2) are thought to be involved. Interspecies transmission of rotavirus to humans has also been indicated by the appearance of unusual G serotypes and VP4 genotypes in humans which are normally limited to a particular animal rotavirus strain (Nakagomi and Nakagomi, 1989; Das *et al.*, 1993).

It should be noted that, unlike circulating strains within the community, rotavirus strains circulating in new-born nurseries often persist for many years with little diversity (Rodger *et al.*, 1981; Perez-Schael *et al.*, 1984).

1.8.1 Serotypic diversity in developed countries

Developed countries tend to display a repetitive pattern of rotavirus infection (due to their temperate locations) with peaks occurring in the cooler months of the year (Kapikian *et al.*, 1976). Epidemiological studies by Masendycz *et al.* (1994) in Australia showed that the serotypic diversity increases as the rotavirus season advances and that major cities have a greater diversity of serotypes than rural communities. In the United States, rotavirus diarrhoea is seen to spread yearly starting from Mexico (and the Southwest) and ending in the Northeast (Ho *et al.*, 1988).

In the 1970's the G2 serotype was predominant in Australia (Bishop *et al.*, 1991) and was thought to be predominant in much of Europe during this time (Maunula and Bonsdorff, 1995). However, the G1 serotype has predominated in most of the developed world during the 1980's/early 1990's (Beards *et al.*, 1989; Gouvea *et al.*, 1990; Bishop *et al.*, 1991; Maunula and Bonsdorff, 1995) although it should be noted that the G1 subtype varies seasonally (Bishop *et al.*, 1991). Epidemic occurrences of the G4 serotype are common in the developed world (Beards *et al.*, 1989; Maunula and Bonsdorff, 1995) and an Australia study showed that the G4 serotype had an episodic pattern of appearance, with peak incidences approximately every three years (Bishop *et al.*, 1991). The G2 and G3 serotypes have generally been the least prevalent serotypes in the developed world during the 1980's/early 1990's and unpredictable in occurrence (Beards *et al.*, 1989; Bishop *et al.*, 1991; Maunula and Bonsdorff, 1995). Conversely, the G3 serotype was the most prevalent serotype in Houston during the 1987-1988 season, although, the G1

serotype was predominant in all the other American cities studied at that time (Gouvea *et al.*, 1990; O'Ryan *et al.*, 1990).

Initial studies on the distribution of VP4 genotypes indicated that VP4 genotype 8 was the most frequent, followed by genotype 4, among 400 samples collected from many areas of the world (Steele *et al.*, 1993). Recently, studies by Santos *et al.* (1994) found that the G1 serotype (VP4 genotype 8) predominated in the United States followed closely by the G3 serotype (VP4 genotype 8).

1.8.2 Serotypic diversity in developing countries

In developing countries, the G1 to G4 serotypes are distributed more equally than in developed countries, albeit, that the G1 serotype is again often predominant (Gomez *et al.*, 1990; Padilla-Noriega *et al.*, 1990). Similarly to the situation found in developed countries, the G3 or G4 serotypes can be predominant in developing countries (Brussow *et al.*, 1990; Timenetsky *et al.*, 1994).

Natural rotavirus coinfections appear to be more frequent in developing countries than in the more developed countries, for example, from 1986 to 1992, nineteen percent of patients showed coinfections in Brazil compared with just 1% of patients in Hungary (Timenetsky *et al.*, 1994; Szucs *et al.*, 1995). This may be due to lower levels of hygiene and frequent overcrowding in the developing countries. In addition, the appearance of G serotypes and VP4 genotypes in humans which are normally limited to a particular animal rotavirus strain (Nakagomi and Nakagomi, 1989; Das *et al.*, 1993) has been speculated to be a result of interspecies transmission. Indeed it is thought that interspecies transmission may occur more frequently in developing countries due to a closer contact of humans with animals. Consequently, it has been suggested that genetic reassortment within the host and interspecies transmission leads to the complex serotypic diversity of rotavirus strains seen in developing countries.

The serotypic diversity of rotavirus strains in developing countries has been observed in both the G serotypes and VP4 genotypes. For example, in addition to the G1 to G4 serotypes, the G5 serotype was found to be associated with diarrhoea in Brazilian children during the 1980's (Gouvea *et al.*, 1994) and an asymptomatic rotavirus G9 serotype was predominant between 1986 and 1993 in new-borns from New Delhi, India (Das *et al.*, 1994). VP4 genotype 8 is believed to be the most prevalent, followed by VP4 genotype 4, in many areas of the world (Steele *et al.*, 1993), however, unusual VP4 genotypes 6 and 11 (both serotype G9) were predominant in new-borns from New Delhi, India between 1986 and 1993 (Das *et al.*, 1994). In addition, although VP4 genotype 8 (in association with serotypes G1 and G3) was predominant in Sao Paulo, Brazil from 1986 to 1992, VP4 genotypes 3, 4 and 6 were frequently linked with disease and each was associated with at least two different G1 to G4 serotypes (Timenetsky *et al.*, 1994).

CHAPTER 2

THE CTL AND ITS ROLE IN THE IMMUNE RESPONSE

2.1 INTRODUCTION

Major histocompatibility (MHC) class I and class II molecules are highly polymorphic cell surface proteins which present antigenic peptides to the thymus-educated (T)-lymphocytes of the immune system. They allow the differentiation of 'self' from 'non-self' so that defence mechanisms are normally directed towards virus infected cells, tumours, transplants and other foreign molecules without causing damage to host tissues (Madden, 1995). Naive T-lymphocytes are educated to differentiate between MHC molecules containing peptides derived from 'self' or 'non-self' proteins by processes of positive and negative selection in the thymus (Allen, 1994). The two classes of MHC are similar at both the genomic and structural level (Jackson and Peterson, 1993; Wolf and Ploegh, 1995). However, MHC class I molecules are found on most cell types and express peptides derived from intracellular proteins (including viral proteins) (Jackson and Peterson, 1993), whereas, MHC class II molecules are restricted to bursa-derived (B)-lymphocytes (antibody producing) and specialised antigen presenting cells (APCs), and express peptides derived from endocytic compartments (Wolf and Ploegh, 1995).

Cytotoxic T-lymphocytes (CTLs), which express the $CD3^+$ and $CD8^+$ clusters of differentiation (CD) molecules, only recognise antigen in association with MHC class I molecules. CTLs destroy cells displaying 'non-self' antigens and are, therefore, central in combating intracellular pathogens (Jackson and Peterson, 1993). The other major subset of T-lymphocytes, T helper (Th)-lymphocytes, which express $CD3^+$ and $CD4^+$, only recognise antigen in association MHC class II molecules. Th-lymphocytes are regulatory cells which control the development and function of effector cells (Mosmann and Coffman, 1989). In some cases, however, the functions of the T-lymphocyte subsets overlap. CTLs are capable of secreting cytokines and controlling B-lymphocyte antibody production *in vitro* (Erard *et al.*, 1993) and Th-lymphocytes can lyse target cells utilising a

perforin-dependent, antigen-specific cytotoxic mechanism (Williams and Engelhard, 1996).

2.2 OVERVIEW OF T-LYMPHOCYTES IN VIRAL INFECTION

2.2.1 CTLs are critical for clearing many viral infections

The immune response to virus infection is still not completely understood and the underlying mechanisms leading to virus clearance are still under debate. Indeed, this is not surprising considering the fact that viruses differ greatly in their route of entry, site of replication, and escape of immune surveillance and release. The cell-mediated immune response to viruses is considered to be very important in the clearance of many primary viral infections. Studies have shown that the adoptive transfer of virus-specific CTLs into a host infected with homologous virus is sufficient to clear virus infection. For example, transferred CTLs appeared sufficient to clear murine cytomegalovirus (MCMV) from the lungs of immunocompromised mice (Reddehase *et al.*, 1985/1987b) and prevent mortality of normal mice from high virulence strains of influenza virus (Bender *et al.*, 1992). Transferred CTLs were also speculated to prevent cytomegalovirus (CMV) infection in immunodeficient humans (Riddell *et al.*, 1992). The influenza virus-specific CTL response has been shown to be subtype-independent *in vitro* and to contribute to heterotypic virus clearance *in vivo* (Biddison *et al.*, 1979; Liang *et al.*, 1994). Serotype-independent CTL responses have been found in many virus systems including respiratory syncytial virus (RSV), lymphocytic choriomeningitis virus (LCMV) and human immunodeficiency virus 1 (HIV-1) (Bangram and Askonas, 1986; Whitton *et al.*, 1988a/b; Casement *et al.*, 1995).

The important role of CTLs in controlling virus infection was highlighted by studies on HIV-1 infection in humans. The transient control of HIV-1 replication in the early clinical latent phase correlates to a vigorous polyclonal $CD8^+$ CTL response (Carmichael *et al.*, 1993: Borrow *et al.*, 1994). However, both the HIV-1-specific CTL and

the Th-lymphocyte count is decreased in patients with acquired immunodeficiency syndrome (AIDS) or AIDS-related complex (ARC) (Carmichael *et al.*, 1993).

Recently, two types of transgenic mice have been used to study the CTL response to viral infections more closely *in vivo*, (i) β_2-microglobulin (β_2m) knockout mice, which fail to express class I proteins and are virtually devoid of CTLs and (ii) perforin deficient mice, which produce virus-specific CTLs deficient in cytotoxicity. The importance of the CTL response to LCMV infection was confirmed by the development in such knockout mice of a chronic LCMV infection (Lehmann-Grube *et al.*, 1993; Kagi *et al.*, 1994a). β_2m-knockout mice also showed delayed clearance of both Sendai virus (Hou *et al.*, 1992) and low virulence influenza virus (Bender *et al.*, 1992). On the contrary, both types of transgenic mice cleared vaccinia virus infection normally (Spriggs *et al.*, 1992; Kagi *et al.*, 1995) and perforin deficient mice infected with either vesicular stomatitis virus (VSV) or Semliki forest virus (SFV) did not show increased mortality compared to normal mice (Kagi *et al.*, 1995).

These results suggest that although CTLs are important in the clearance of virus infections, systemic viruses which lead to the cytopathology of infected cells prior to their recognition by CTLs, are cleared with the help of other immune mechanisms. However, viruses differ greatly in both their site of replication (systemic or mucosal) and rate of cell cytopathology. Therefore, the exact contributions of the various cells of the immune system probably depends very much on the individual virus type. In addition, the plasticity of the immune system is speculated to compensate if virus-specific CTLs are absent (or non-functional) throughout the development of the immune response to a virus. Cells which have been suggested to help clear virus infection of mice in the absence of CTLs include antibody producing B-lymphocytes, cytokine [interferon (IFN)-γ and tumour necrosis factor (TNF)] producing lymphocytes, Th-lymphocytes, $\gamma\delta$ T-lymphocytes and natural killer (NK) cells.

2.2.2 The role of Th-lymphocytes in clearing viral infection

Th-lymphocytes are thought to mediate viral clearance through the production of cytokines which act either directly to inhibit virus replication, or locally and systemically to regulate other components of the host's immune response. Murine Th-lymphocytes have been divided into three subsets (Th0, Th1 and Th2) depending on their cytokine profiles (Mosmann *et al.*, 1986; Paliard *et al.*, 1991).

Th0-lymphocytes have a cytokine profile which overlaps Th1 and Th2-lymphocytes and were speculated to be the precursor of the two subsets (Paliard *et al.*, 1991). Depending on the predominant cytokines in the milieu of Th0-lymphocytes, they differentiate and/or selectively outgrow into either a Th1 [promoted by IFN-γ and interleukin (IL)-12] or Th2 (promoted by IL-4 and IL-10) lymphocyte population (Mosmann and Moore, 1991; Maggi *et al.*, 1992; Hsieh *et al.*, 1993).

Th1-lymphocytes produce IL-2, IFN-γ and TNF and these preferentially stimulate cell-mediated immune responses, including CTLs and delayed type hypersensitivity (DTH) (Cher and Mosmann, 1987). IL-2 is a major growth factor for T and B-lymphocytes, activates NK cells and promotes B-lymphocyte differentiation (Ferrar *et al.*, 1982). IFN-γ and TNF activate macrophages and NK cells (Staeheli, 1990), directly inhibit virus replication (Feduchi and Carrasco, 1991), promote resistance in uninfected cells to viral infection (Wong and Goeddel, 1986) and have showed direct antiviral activity (Kohonen-Corish *et al.*, 1990; Sambhi *et al.*, 1991). In addition, IFN-γ can also promote CTL recognition of target cells by increasing MHC class I presentation (Burowski and Welsh, 1985). Because IL-2 is essential for the effective generation and expansion of a virus-specific CTL response (Reddehase *et al.*, 1987a) and because IFN-γ and TNF mediate various antiviral activities, the Th1-lymphocyte response is thought to be the most beneficial in resolving viral infections.

By contrast, Th2-lymphocytes produce IL-4, IL-5, IL-6 and IL-10 and these in turn preferentially stimulate antibody-mediated immune responses, and activate eosinophils and mast cells (Coffman *et al.*, 1988). It should be noted that virus-specific antibody is important in limiting viral spread (Koszinowski *et al.*, 1991) and is likely to have a significant role in the protection from re-infection of some viruses (Kagi *et al.*, 1995). A summary of the role of $CD4^+$ Th-lymphocytes in the immune response is shown in Figure 2.

2.3 THE STRUCTURE AND FUNCTION OF THE MHC CLASS I MOLECULE

2.3.1 The genes encoding the mouse MHC class I proteins

The mouse MHC genes are situated in the H-2 region of chromosome 17 (see Figure 3). There are approximately 30 class I genes in the haploid mouse genome. At least three class I genes encode the classical and highly polymorphic transplantation antigens, H-2K, H-2D and H-2L. Two class I genes K and K1 are situated in the H-2K region in a head-to-tail configuration and their expression depends on the particular mouse strain. The organisation of the H-2K region is similar in Balb/c ($H\text{-}2^d$), C57BL/6 ($H\text{-}2^b$) and C3H/He,mg ($H\text{-}2^k$) mice with the expression of $H\text{-}2K^d$, $H\text{-}2K^b$ and $H\text{-}2K^k$, respectively (Hood *et al.*, 1983; Weiss *et al.*, 1984; Flavell *et al.*, 1986). The number of genes not only present but also expressed in the D/L region varies between different haplotypes of mice. $H\text{-}2^d$ mice have five genes in this region but only two of them ($H\text{-}2D^d$ and $H\text{-}2L^d$) are expressed. The functions of the three silent genes ($D2^d$, $D3^d$ and $D4^d$) are not known. By contrast, $H\text{-}2^b$ and $H\text{-}2^k$ mice only have one gene present and expressing in the D/L region, $H\text{-}2D^b$ and $H\text{-}2D^k$ respectively, and these genes are in fact alleles of the $H\text{-}2L^d$ gene in $H\text{-}2^d$ mice. The other protein of the MHC class I complex β_2-microglobulin (β_2m) is encoded outside the H-2 and Qa/Tla complexes and is not polymorphic (Hood *et al.*, 1983; Weiss *et al.*, 1984; Flavell *et al.*, 1986; Watts *et al.*, 1989).

FIGURE 2. THE ROLE OF $CD4^+$ Th-LYMPHOCYTES IN THE IMMUNE RESPONSE

Th0-lymphocytes differentiate into Th1 or Th2-lymphocytes depending on the predominant cytokines in the milieu of the precursor cell. The Th-lymphocyte recognises foreign peptide presented on the surface of antigen presenting cells (APC), in association with the MHC class II molecule (indicated by back arrows). The cytokines secreted by Th1 and Th2 lymphocytes are shown as thick red and blue arrows, respectively, and mutually inhibitory interactions are represented by broken arrows. Thin arrows show the growth and differentiation of different cells of the immune system following stimulation by the relevant Th-lymphocyte cytokines. Antibodies produced by B-lymphocytes can bind to the cellular Fc receptors and induce antibody-dependent cell-mediated cytotoxicity (ADCC). The Th1-lymphocyte response is thought to preferentially stimulate a cell-mediated immune response, whereas, the Th2-lymphocyte response is thought to preferentially stimulate an antibody-mediated immune response.

Modified from Male *et al.* (1991).

FIGURE 2

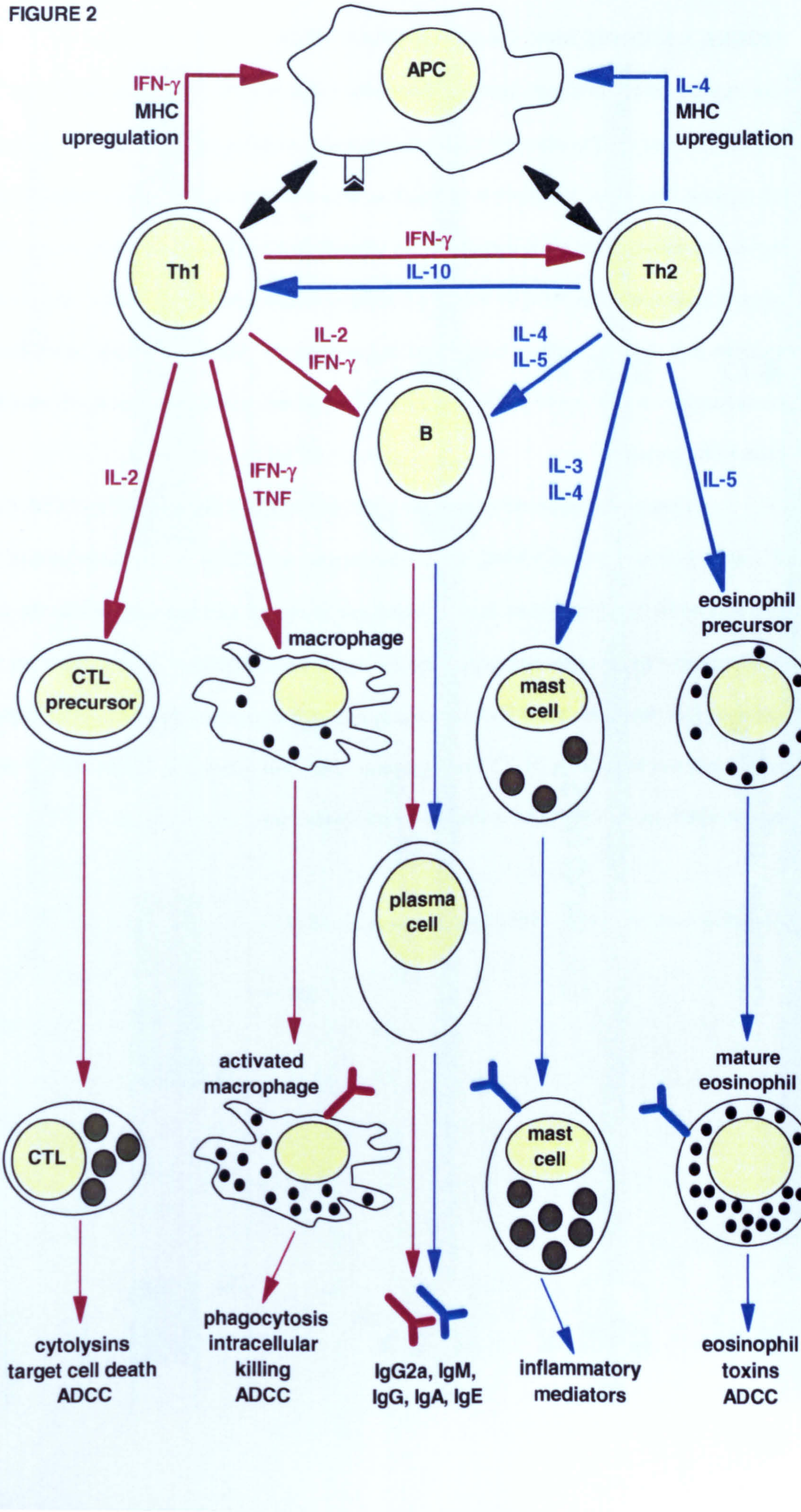

APC
IFN-γ
MHC
upregulation
IL-4
MHC
upregulation
Th1
Th2
IFN-γ
IL-10
IL-2
IFN-γ
IL-4
IL-5
B
IL-2
IFN-γ
TNF
IL-3
IL-4
IL-5
eosinophil
precursor
macrophage
CTL
precursor
mast
cell
plasma
cell
activated
macrophage
mature
eosinophil
CTL
cytolysins
target cell death
ADCC
phagocytosis
intracellular
killing
ADCC
IgG2a, IgM,
IgG, IgA, IgE
mast
cell
inflammatory
mediators
eosinophil
toxins
ADCC

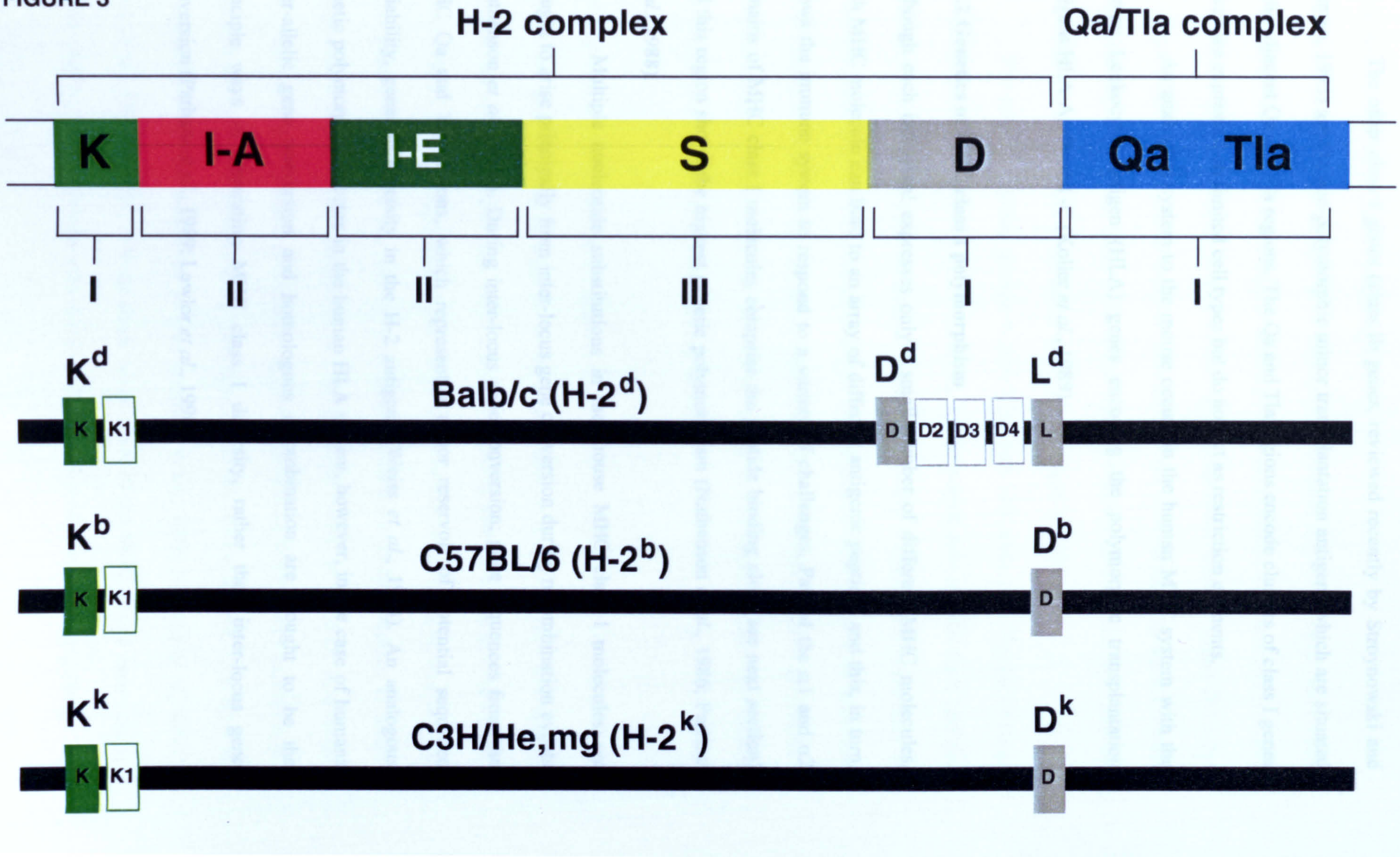
FIGURE 3. MURINE MHC CLASS I HAPLOTYPES

The upper part of the figure shows a schematic representation of the organisation of the murine H-2 and Qa/Tla complexes. The H-2 complex consists of class I, class II and class III regions. The class I regions K (in light green) and D (in grey) encode MHC class I molecules whereas, the class II regions I-A (in red) and I-E (in dark green) encode MHC class II molecules. The class III region (in yellow) resides between the class I and class II regions and consists of a heterogeneous mixture of genes including complement components and TNF α and β. The murine Qa/Tla regions (in blue) contain genes encoding class I-like proteins.

A schematic representation of the genes of the mouse Balb/c (H-2^d), C57BL/6 (H-2^b) and C3H/He,mg (H-2^k) MHC class I haplotypes are shown in the lower part of the figure. The MHC class I genes in each haplotype of mouse are shown by boxes, the solid boxes represent the expressed genes and the open boxes represent genes which are not expressed. It should be noted that all three mouse strains express the H-2K gene in the K region and that the Balb/c (H-2^d) strain contains additional genes (D2, D3 and D4) in the D region which are thought to have arisen by gene duplication.

Modified from Male *et al.* (1991) and Watts *et al.* (1989).

FIGURE 3


H-2 complex
Qa/Tla complex
K
I-A
I-E
S
D
Qa
Tla
I
II
II
III
I
I
K^d
Balb/c ($H\text{-}2^d$)
D^d
L^d
K
K1
D
D2
D3
D4
L
K^b
C57BL/6 ($H\text{-}2^b$)
D^b
K
K1
D
K^k
C3H/He,mg ($H\text{-}2^k$)
D^k
K
K1
D

The other class I genes (class Ib genes, reviewed recently by Stroynowski and Forman, 1995) are the non-polymorphic minor transplantation antigens which are situated in the adjacent Qa and Tla regions. The Qa and Tla regions encode clusters of class I genes which are expressed on limited cell types but do not act as restriction elements.

An analogous system to the mouse occurs in the human MHC system with the Human Leukocyte Antigen (HLA) genes encoding the polymorphic transplantation antigens HLA-A, -B, and -C (Koller *et al.*, 1989).

2.3.2 Genetics of MHC class I polymorphism

Although each individual expresses only a small number of different MHC molecules, each MHC molecule can bind to an array of different antigenic peptides and this, in turn, allows the immune system to respond to a variety of challenges. Part of the $\alpha 1$ and $\alpha 2$ domains of MHC class I molecules comprise the peptide binding cleft (see next section) and this region shows the highest genetic polymorphism (Nathenson *et al.*, 1986; Parham *et al.*, 1988).

Multiple nucleotide substitutions in the mouse MHC class I molecules are thought to arise principally from inter-locus gene conversion during recombination events (Nathenson *et al.*, 1986). During inter-locus gene conversion; gene sequences from the MHC Qa and Tla regions, which represent a donor reservoir of potential sequence variability, generate diversity in the H-2 antigens (Weiss *et al.*, 1983). An analogous genetic polymorphism occurs in the human HLA system, however, in the case of humans inter-allelic gene conversion and homologous recombination are thought to be the principle ways of generating MHC class I diversity, rather than inter-locus gene conversion (Parham *et al.*, 1989; Lawlor *et al.*, 1990).

2.3.3 Structure of MHC class I molecules

MHC class I molecules consist of a highly polymorphic type I integral membrane glycoprotein heavy chain (46 kDa), containing three extracellular domains (α1, α2 and α3), noncovalently associated with a soluble β_2-microglobulin subunit (β_2m) (12 kDa) (see Figure 4A). The α1 and α2 domains provide both the α helices and an eight stranded antiparallel β-pleated sheet floor to form the antigen binding groove. The β_2m domain binds to all three α domains of the heavy chain (Bjorkman *et al.*, 1987a/b).

The peptide binding groove is 30Å long and 12Å wide (see Figure 4B) and contains pockets, separated by fixed distances, which extend between the floor and the helical walls of the α1 and α2 domains (Garrett *et al.*, 1989). The amino acid residues of the peptide binding groove α helices (which are highly polymorphic) determine the shape of the binding site and, therefore, the peptides bound by each allelic protein. In contrast, the amino residues which block the ends of the MHC class I peptide groove are highly conserved (Matsumura *et al.*, 1992; Madden, 1995).

2.3.4 Peptides bound by the MHC groove

Peptides and glycopeptides of eight to eleven amino acids in length are held in a largely extended conformation which runs the length of the binding groove (Fremont *et al.*, 1992; Madden, 1995). Rarely, longer peptides can bind with one termini hanging beyond the end of the groove (Collins *et al.*, 1994). The carboxy terminus of the peptide is either hydrophobic or charged (Rammensee *et al.*, 1993) and is buried in the cleft to a lesser extent than the amino terminus (Fremont *et al.*, 1992). Although there is a prominent arch which lifts the peptide main chain away from the floor of the binding groove, 73 to 83% of the peptide is concealed within the groove (Fremont *et al.*, 1992; Madden *et al.*, 1993; Young *et al.*, 1994). Peptide binding alters the structure of the MHC class I heavy chain and the resulting structure varies with the type of peptide bound (Fremont *et al.*, 1992).

FIGURE 4. STRUCTURE OF A MHC CLASS I MOLECULE

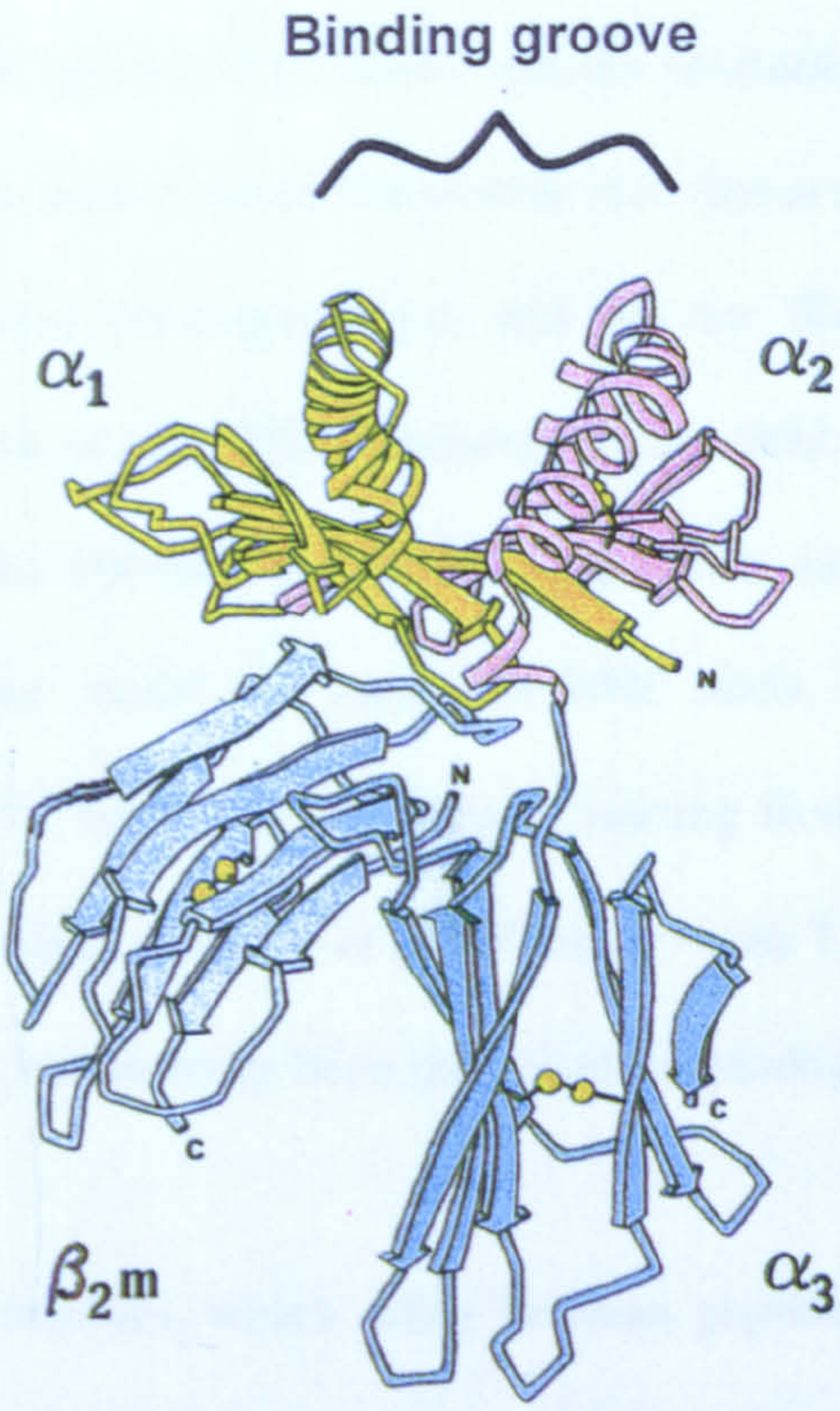
A schematic representation of the human HLA-A2 MHC class I molecule (Bjorkman *et al.*, 1987a). Thick arrows represent β-strands in the amino to carboxyl direction, helical ribbons represent α-helices and two connected circles represent disulphide bonds.

(A). A side view of the MHC class I molecule showing the highly polymorphic α1 and α2 domains (the peptide binding site) and the membrane proximal α3 and β_2-microglobulin (β_2m) domains. The α1 and α2 domains consist of an eight stranded β-pleated sheet covered by α-helices to form the peptide binding groove.

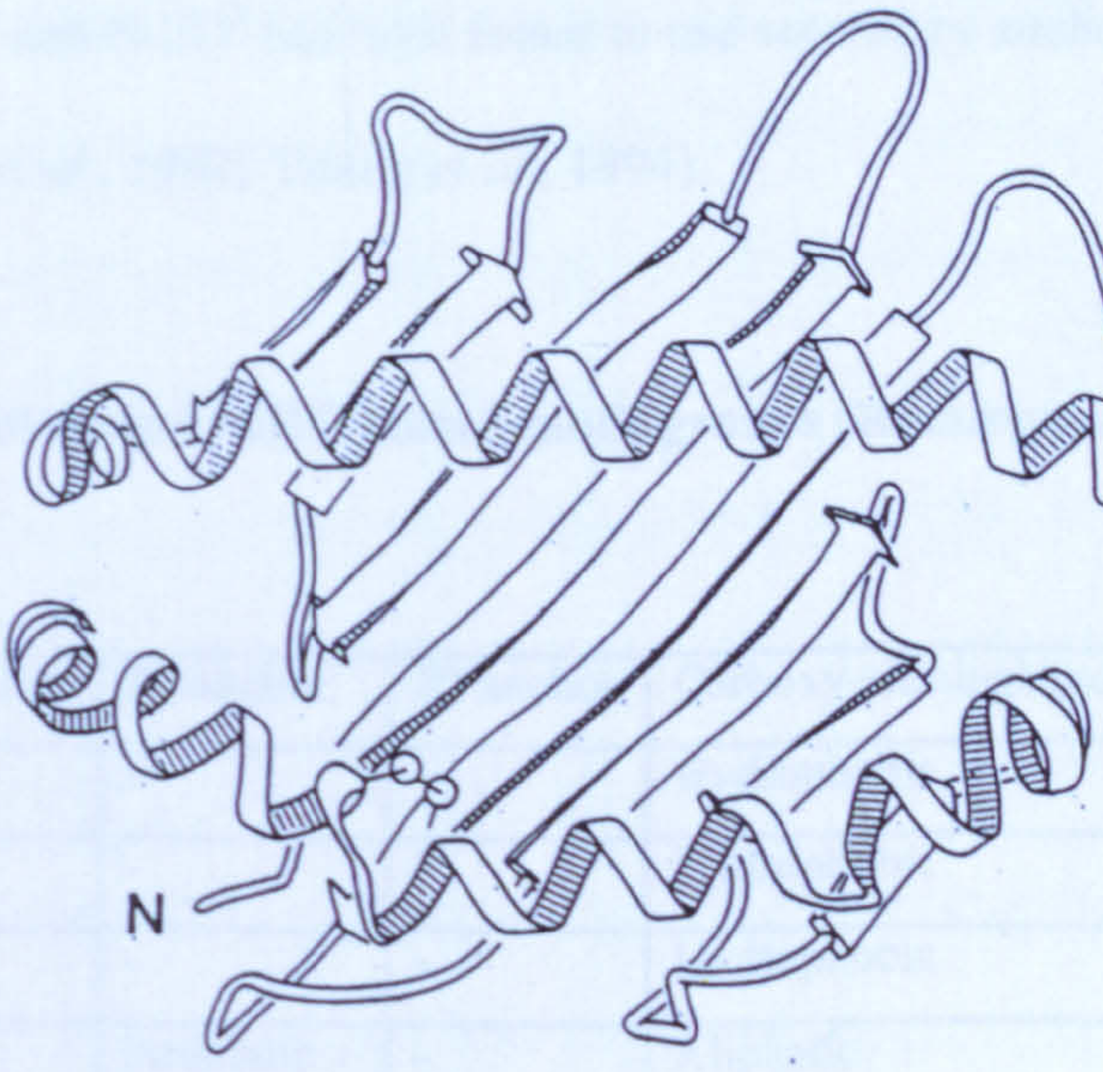
(B). Top view of the MHC class I molecule (α1 and α2) [90° from Figure 4 (A)] showing the peptide binding groove. The N terminus of the α1 domain is shown. The α1 and α2 domains both consist of four anti-parallel β-strands followed by a α-helical region. The α1 and α2 domains pair to form the eight stranded β-pleated sheet covered by α-helices.

FIGURE 4

(A)



(B)



Thousands of different peptides, providing they adopt the required backbone conformation, can bind to one MHC allele because only a few positions on the peptide actually bind to the MHC groove. Non-allelic specific interactions at the peptide termini and allelic specific interactions between the peptide side chains and the MHC side pockets provide the bonding forces (hydrogen bonds and van der Waals bonds) of the MHC-peptide complex (Fremont *et al.*, 1992 Matsumura *et al.*, 1992; Young *et al.*, 1994). The allele specific interactions between the peptide side chains and the side pockets of the MHC provide the binding 'motif' of a particular MHC allele. In designating residues, P denotes the position of the residue in the peptide, starting from the amino-terminal end. Binding occurs at P2 in many cases, or at P3, P5 and P7 (see Table 2). A detailed review of MHC binding motifs has recently been published by Madden, 1995 and Rammensee, 1995.

Other peptide residues, which differ between peptides, can also make contact with MHC "loose" pockets. Within a particular MHC motif, certain non-anchor peptide residues are preferentially used or not used, and are involved in the fine tuning of the peptide specificity (Ruppert *et al.*, 1993). For example, peptide bound to the mouse MHC class I alleles H-2K^b and H-2D^b has been found to use secondary anchors in the P3 binding pocket (Matsumura *et al.*, 1992; Young *et al.*, 1994).

Table 2. Examples of mouse MHC class I motif groups (Rammensee, 1995)

P2 anchor	P3 anchor	P5 anchor	P7 anchor	Carboxy-terminal anchor	MHC allele
Tyr	-	-	-	Hydrophobic	H-2K^d
Pro	-	-	-	Hydrophobic	H-2L^d
Negative	-	-	-	Hydrophobic	H-2K^k
-	-	Aromatic	-	Aliphatic	H-2K^b
-	-	Asn	-	Aliphatic	H-2D^b

Although peptides bind to MHC using only a few amino acid residues, the overall sequence of the peptide affects its main-chain conformation, the orientation of its side chains and, thus, the antigenic uniqueness of the resulting MHC-peptide complex (Fremont *et al.*, 1992; Madden *et al.*, 1993). The peptide also contains "flag" side chains which are orientated towards the T-cell receptor (TCR) and so could, therefore, affect TCR recognition (Silver *et al.*, 1992).

2.4 ASSEMBLY AND TRANSPORT OF MHC CLASS I MOLECULES

2.4.1 Generation of MHC class I components and transport into the ER

It is believed that MHC class I heavy chains and β_2m are both translated, with a signal sequence, in the cytoplasm of the antigen presenting cell (APC). The signal sequences of the nascent polypeptides directs them to the endoplasmic reticulum (ER) where, following translocation into the ER, the signal sequences are cleaved by the resident ER signal peptide peptidase (Germain and Marguiles, 1993; Jackson and Peterson, 1993).

2.4.2 Generation of peptides and transport into the ER

Peptides for MHC binding are thought to be derived predominantly from nascent polypeptides generated in the cytoplasm of the APC (Moore *et al.*, 1988; Yewdell *et al.*, 1988). The multi-catalytic protein protease (proteasome) is abundant in both the cytoplasm and nucleus of the cell and looks increasingly convincing as the key protease in the generation of peptides for class I molecules (Goldberg and Rock, 1992). Proteasomes are composed of more than 25 polypeptide subunits (total molecular weight approximately 2000 kDa) and have been linked to an ATP-dependent proteolytic pathway in which proteins are initially marked for degradation following binding with the ubiquitin protein (Peters, 1994). Two of its β subunits, low molecular weight polypeptides (LMP)2 and LMP7, are encoded by genes within the mouse and human MHC (Brown *et al.*, 1991;

Glynne *et al.*, 1991; Kelly *et al.*, 1991; Martinez and Monaco, 1991). LMP2 and LMP7 are induced by IFN-γ but are not essential for normal peptide generation (Zhou *et al.*, 1994). Following IFN-γ induction, LMP2 and LMP7 replace the constitutive and highly homologous proteasome β subunits (which are also down regulated by IFN-γ) (Fruh *et al.*, 1994; Belich *et al.*, 1994). This replacement is speculated to narrow the proteasome's polypeptide cleaving properties so that the proteasome preferentially provides 'finished' peptides for MHC class I binding (Gaczynska *et al.*, 1994).

Peptides enter the ER using an ATP-dependent, transporter associated with the antigen processing (TAP) pathway (Neefjes *et al.*, 1993; Shepherd *et al.*, 1993) and an ATP-independent pathway of unknown identity (Dobberstein, 1992). The TAP heterodimer is a typical ATP-binding cassette transporter which, like LMP2 and LMP7, is encoded by genes within the mouse and human MHC (Monaco *et al.*, 1990; Spies *et al.*, 1990; Trowsdale *et al.*, 1990). TAP favours the transport of peptides which are the correct size for MHC class I loading (Momburg *et al.*, 1994a; Schumacher *et al.*, 1994), however, longer peptides (up to 33 amino acids long) also appear to be dependent on TAP for transport into the ER (Urban *et al.*, 1994; Neisig *et al.*, 1995). There is also evidence to suggest that TAP shows specificity for the carboxyl termini amino acid of the peptides it transports. Experiments by Momburg *et al.* (1994b) showed that this specificity varies between different species, mouse TAP being restricted to the transport of peptides with hydrophobic carboxy termini, whereas, human TAP can transport all carboxy-terminal amino acids except proline and possibly glycine. Generally, the experiments on TAP suggest that it preferentially transports 'finished' peptides for MHC class I binding.

Recent work suggests that most viral proteins which cross directly into the ER, via their signal sequences, cannot associate with MHC class I molecules (Hammond *et al.*, 1995; Hombach *et al.*, 1995). The authors suggest that these viral proteins are mislocalised

to the cytosol where they then enter the supply and transport of peptides presented by MHC class I molecules.

2.4.3 Association of MHC class I and peptides

Within the biosynthetic compartment of the ER, the MHC class I heavy chains transiently bind to resident ER membrane chaperone proteins, including calnexin and immunoglobulin binding protein (BiP) (Nößner and Parham, 1995). It has been speculated that subsequent β_2m binding to the MHC heavy chain results in the dissociation of the chaperone (Ortmann *et al.*, 1994; Nößner and Parham, 1995). It then appears that the heterodimers bind to components of the TAP protein within the ER (Ortmann *et al.*, 1994; Suh *et al.*, 1994). TAP is believed to deliver a peptide to the heterodimer which, following its stabilisation, dissociates from the TAP protein (Suh *et al.*, 1994). Finally, the MHC-peptide is transported through the Golgi apparatus to the cell surface (Jackson and Peterson, 1993). A summary of the generation of peptides and subsequent MHC class I binding is shown in Figure 5.

2.4.4 Antigen presenting cells (APCs)/target cells

Although MHC class I molecules are expressed by the majority of human cell types (Male *et al.*, 1991), APCs can be divided into both professionals and amateurs (Sprent, 1995). Professional APCs, such as macrophages and dendritic cells, express higher levels of co-stimulatory molecules than amateur APCs (Steinman, 1991; Schwartz, 1992). Professional APCs are scattered throughout the body but are concentrated particularly in T-lymphocyte-dependent areas of the lymphoid tissues, such as the spleen and lymph nodes. These cells are believed to acquire (and process) antigen and transport it to the lymphoid tissues (Macatonia *et al.*, 1989; Hill *et al.*, 1990). It is also possible that free virus particles or proteins could enter the afferent lymph and be acquired by professional APCs resident

FIGURE 5. A SCHEMATIC REPRESENTATION OF MHC CLASS I ASSEMBLY AND PEPTIDE PROCESSING

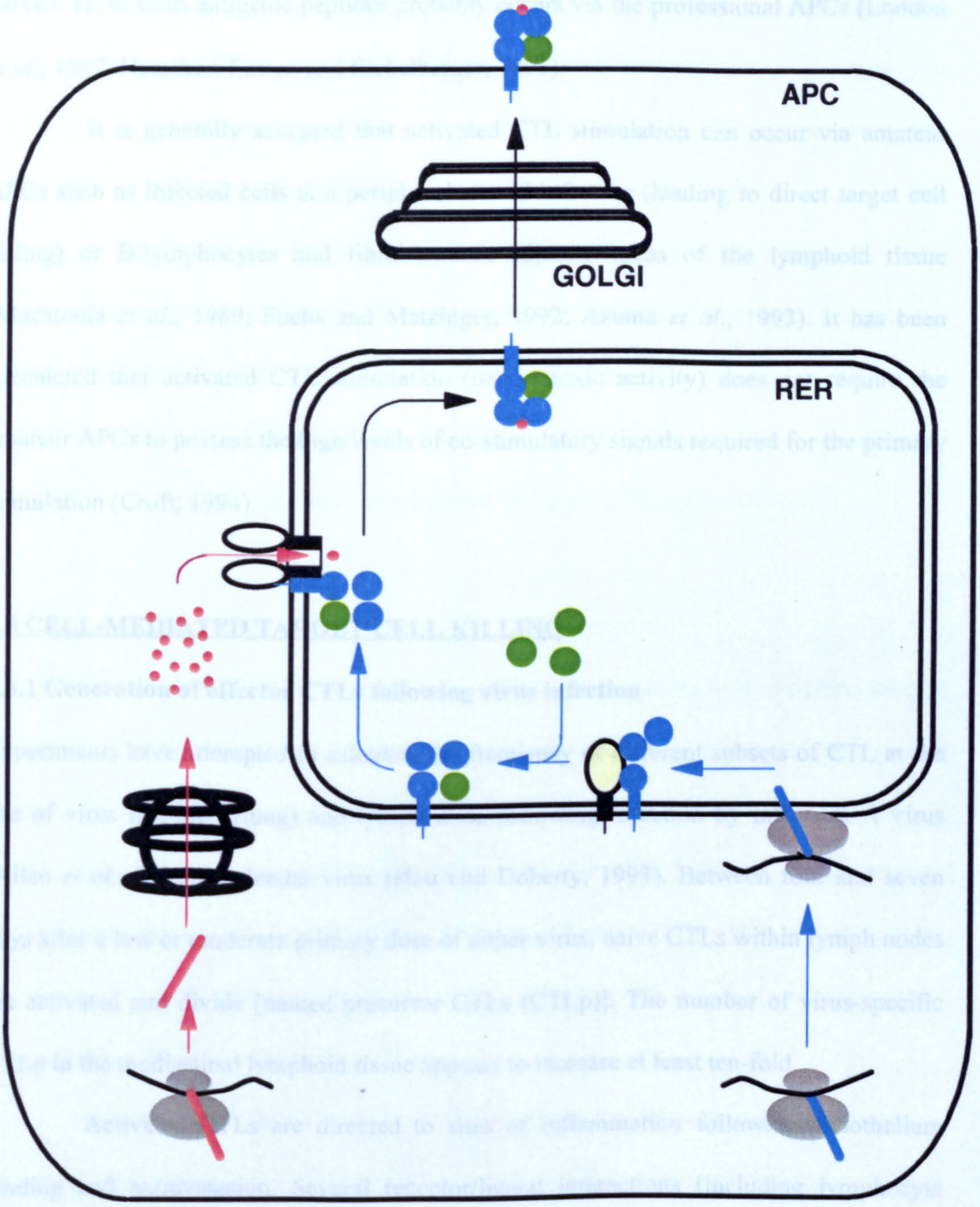
MHC class I heavy chains and β_2-microglobulin (β_2m) are both translated in the cytoplasm of the antigen presenting cell (APC) and signal sequences direct the nascent polypeptides into the lumen of the endoplasmic reticulum (ER). The signal sequences are cleaved by resident ER signal peptide peptidases and the class I heavy chains bind to ER membrane chaperone proteins, including calnexin and immunoglobulin binding protein (BiP). It has been speculated that the binding of the β_2m to the class I heavy chains triggers the dissociation of the heterodimer from the chaperone.

Peptides for MHC class I binding are derived predominantly from nascent polypeptides translated in the cytoplasm of the APC. It is believed that the multi-catalytic protein protease (proteasome) generates the peptides for MHC class I presentation in the cytoplasm of the cell. The majority of these peptides are thought to enter the lumen of the ER via the transporter associated with the antigen processing (TAP transporter) pathway.

Transported peptides are believed to load into class I molecules that are physically tethered to the TAP transporter. Binding of the peptide triggers stabilisation and, consequently, release of the MHC-peptide from the TAP transporter. The newly formed MHC-peptide is transported through the Golgi apparatus to the cell surface where CTLs examine it for peptides derived from foreign proteins.

Modified from Jackson and Peterson (1993).

FIGURE 5


APC
GOLGI
RER
Ribosomes producing nascent polypeptide
β_2 microglobulin
Peptide generated by cytosolic proteases
Class I heavy chain 3 unfolded domains
Class I heterodimer unstable
TAP transporter
ER membrane chaperone proteins: calnexin and BiP
Proteasome
MHC class I presenting peptide

within the lymph nodes (Eichelberger *et al.*, 1991). At these sites, primary stimulation of naive CTL to virus antigenic peptides probably occurs via the professional APCs (London *et al.*, 1987; Hamilton-Easton and Eichelberger, 1995).

It is generally accepted that activated CTL stimulation can occur via amateur APCs such as infected cells at a peripheral site of infection (leading to direct target cell killing) or B-lymphocytes and fibroblasts in adjacent areas of the lymphoid tissue (Macatonia *et al.*, 1989; Fuchs and Matzinger, 1992; Azuma *et al.*, 1993). It has been speculated that activated CTL stimulation (or cytotoxic activity) does not require the amateur APCs to possess the high levels of co-stimulatory signals required for the primary stimulation (Croft, 1994).

2.5 CELL-MEDIATED TARGET CELL KILLING

2.5.1 Generation of effector CTLs following virus infection

Experiments have attempted to calculate the frequency of different subsets of CTL at the site of virus infection (lung) and lymph node following infection by influenza A virus (Allan *et al.*, 1990) or Sendai virus (Hou and Doherty, 1993). Between four and seven days after a low or moderate primary dose of either virus, naive CTLs within lymph nodes are activated and divide [named precursor CTLs (CTLp)]. The number of virus-specific CTLp in the mediastinal lymphoid tissue appears to increase at least ten-fold.

Activated CTLs are directed to sites of inflammation following endothelium binding and extravasation. Several receptor/ligand interactions [including lymphocyte functional antigen (LFA)-1/intercellular adhesion molecule (ICAM)-1 and very late antigen (VLA)-4/vascular cell adhesion molecule (VCAM)-1] have been implicated in this role (reviewed in Shimizu *et al.*, 1992). It appears that the receptors on activated CTL, and the ligands on endothelial cells at the sites of inflammation, are up-regulated (Christensen *et al.*, 1995).

However, recent experiments have speculated that only a minority of the CTLp that localise at the site of infection are specific for the virus and develop into effector CTL (Hou and Doherty, 1993). As explained in the above section, activated CTLs appear to recognise virally infected target cells directly and this results in CTL degranulation and target cell death (Hahn *et al.*, 1994).

Following elimination of the infected cells (usually within two weeks), activated CTLs traffic in large numbers to the gut (Peyer's patches and lamina propria) and the spleen. In these areas, the CTLs die within a few days and the resulting cellular debris appears to be engulfed by phagocytes (reviewed in Sprent, 1994). However, not all virus-specific CTLp are eliminated and some become 'memory' CTL (see section 2.7).

2.5.2 The structure of the αβ T-cell receptor (TCR) complex

The TCR is a multi-subunit complex on the surface of the majority of T-lymphocytes (see Figure 6). Contained within the TCR is the disulphide linked αβ heterodimer (or in a minority of the T-lymphocyte population, a structurally similar TCR-γδ as described in section 2.6) which is responsible for the recognition of the MHC-peptide complex on the surface of the target cell (Dembic *et al.*, 1986). The diversity of the TCR is due to the α and β chains having numerous V, D and J gene segments which can also undergo gene rearrangement (Davis and Bjorkman, 1988).

The α and β chains contain an immunoglobulin-like extracellular domain, a transmembrane region and a short cytoplasmic tail of five to twelve amino acids in length (Allison and Lanier, 1987). Recently, the crystal structure of the extracellular portion of the β chain was determined at a resolution of 1.7Å and confirmed the immunoglobulin-like nature of the receptor (Bentley *et al.*, 1995). The α/β heterodimer is noncovalently associated to the invariant CD3 γ, δ and two ε chains. These chains are similar to the α and β chains, but have a longer cytoplasmic domain ranging from 40 to 80 amino acids

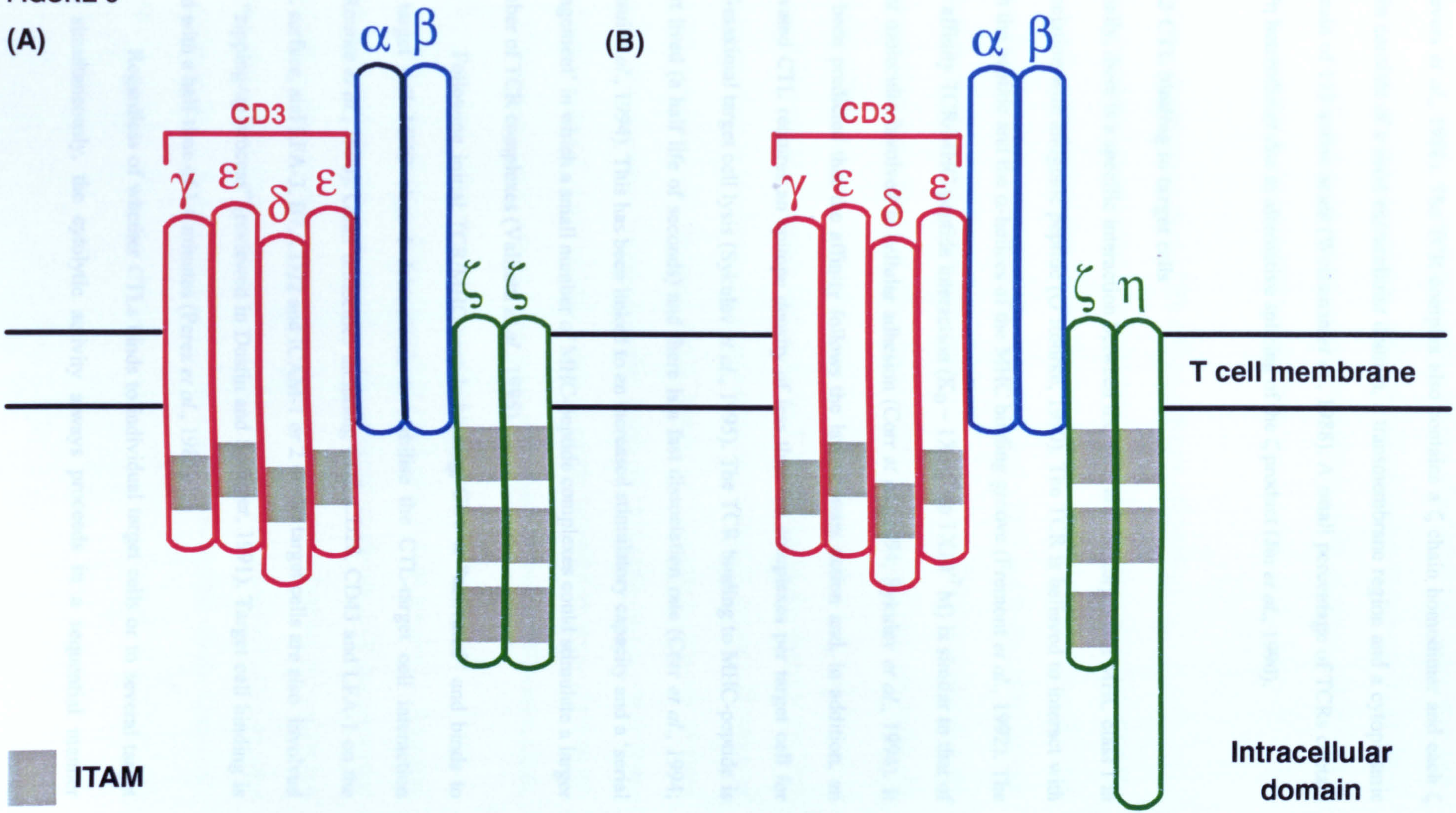
FIGURE 6. THE αβ T CELL RECEPTOR COMPLEX

A schematic representation of the T cell receptor αβ complex showing (A) the common ζ chain homodimer and (B) the small percentage of TCR αβ containing the heterodimer of ζ chain complexed to the η chain.

The TCR binds to MHC-peptide via the α and β chains (in blue) however, extracellular binding events are transduced to the intracellular signalling machinery via both the γ, δ and two ε chains of the CD3 complex (in red) and the ζ homodimer or ζ/η heterodimer chains (in green). The locations of the immunoreceptor tyrosine activation motifs (ITAMs) on the intracellular domains of the γ, δ, ε, ζ and η chains are shown as solid grey boxes (see accompanying text).

Modified from Chan *et al.* (1994) and Bolen (1995).

FIGURE 6


(A)
α β
CD3
γ ε δ ε
ζ ζ
(B)
α β
CD3
γ ε δ ε
ζ η
T cell membrane
ITAM
Intracellular domain

(Clevers *et al.*, 1988). The TCR complex also contains a ζ chain homodimer and each ζ chain consists of a short extracellular domain, a transmembrane region and a cytoplasmic domain of 113 amino acids (Weissman *et al.*, 1988). A small percentage of TCRs contain a ζ/η heterodimer due to alternative splicing of the ζ product (Jin *et al.*, 1990).

2.5.3 CTL binding to target cells

Initially, there is a specific interaction between the TCR and the target cell MHC class I in association with antigenic peptide (O'Rourke, 1990). The TCR is believed to interact with both the peptide and the α-helices of the MHC binding groove (Fremont *et al.*, 1992). The low affinity TCR/MHC-peptide interaction ($K_D \sim 1X10^{-6}$ to $1X10^{-7}$ M) is similar to that of other molecules involved in cellular adhesion (Corr *et al.*, 1994; Sykulev *et al.*, 1994). It has been predicted that the affinity follows the law of mass action and, in addition, an activated CTL requires an epitope density of less than ten complexes per target cell for half-maximal target cell lysis (Sykulev *et al.*, 1995). The TCR binding to MHC-peptide is short lived (a half life of seconds) and there is a fast dissociation rate (Corr *et al.*, 1994; Matsui *et al.*, 1994). This has been linked to an increased stimulatory capacity and a 'serial engagement' in which a small number of MHC-peptide complexes could stimulate a larger number of TCR complexes (Valitutti *et al.*, 1995).

Following initial TCR/MHC-peptide binding, CD8 is "activated" and binds to the target cell MHC class I determinant to stabilise the CTL-target cell interaction (O'Rourke *et al.*, 1990). Other molecules including CD2, CD28, CD43 and LFA-1 on the CTL surface, and LFA-3, B7, CD22 and ICAM-1 or 2 on the target cells are also involved in a "zipping-up process" (reviewed in Dustin and Springer, 1991). Target cell binding is rapid with a half-time of 1.4 minutes (Perez *et al.*, 1985).

Regardless of whether CTLs binds to individual target cells or to several target cells simultaneously, the cytolytic activity always proceeds in a sequential manner

(Zagury, 1982). The CTL detaches following delivery of a "lethal hit" to the target cell, however, the mechanisms involved in detachment are not known. In experiments by Berke *et al.* (1972) CTLs were calculated to undergo multiple cycles of target cell lysis at a constant rate during a five hour *in vitro* incubation.

2.5.4 Signal transduction in the CTL following target cell binding

The mechanisms involved in the CTL signal transduction following target cell binding are only briefly described in this thesis. Recent detailed reviews have been published concerning:- protein tyrosine kinases (PTKs), Chan *et al.* (1994) and Bolen (1995); protein tyrosine phosphatases (PTPs) (and especially CD45), Chan *et al.* (1994) and Okumura and Thomas (1995); immediate early (IE) T-lymphocyte genes and their products, Kelly and Siebenlist (1995).

Following MHC-peptide binding by the TCR α/β chains, the chains of the CD3 and ζ are known to be involved in the signal transduction of the CTL response (Sussman *et al.*, 1988; Letourneur and Klausner, 1992). Within the cytoplasmic domains of these chains are immunoreceptor tyrosine activation motifs (ITAMs) composed of two tyrosine-X-X-leucine (where X is any residue) paired sequences separated by six to eight amino acids. ITAMs occur in triplicate on ζ chains and as a single copy on the CD3 chains (Reth, 1989).

Fyn, Lck and Zap-70 are cytoplasmic PTKs thought to be involved in the signal transduction pathway (Chan *et al.*, 1994). As mentioned previously, CD8 participates in TCR/MHC binding and this molecule also contains a cytoplasmic binding domain for Lck (Turner *et al.*, 1990). CD8 bound Lck is speculated to phosphorylate the ITAMs on the ε and ζ chains of the TCR allowing Zap-70 to subsequently bind to these chains (Chan *et al.*, 1994).

It should be noted that CD45, a member of the family of transmembrane PTP, is an abundantly expressed polymorphic antigen which is required for T-lymphocyte function (Weaver *et al.*, 1991). CD45 is believed to regulate signal transduction pathways in T-lymphocytes by controlling the activities of both Lck and Fyn (Mustelin *et al.*, 1989; 1992)

The activation of Zap-70 results in the tyrosine phosphorylation of a variety of proteins including phospholipase C (Kim *et al.*, 1991) which in turn leads to both an increase in intracellular Ca^{2+} and the activation of protein kinase C (PKC) (Berridge and Irvine, 1984).

This signal cascade eventually lead to the transcription and translation of IE T-lymphocyte genes (Kelly and Siebenlist, 1995). The activation of T-lymphocytes can result in cell proliferation, IL-2 production, induction of cell surface antigens and target cell killing.

2.5.5 CTL induced target cell killing

Following target cell binding, the CTL reorganises its microtubule organising centre (MTOC) and centrioles so that they are proximal to the binding site (Geiger *et al.*, 1982). The microtubules move the Golgi apparatus and lysosomal granules (at rates of up to 3 μms^{-1}) to the vicinity of the contact region probably using the phosphorylation of kinesin-associated proteins (Hahn *et al.*, 1994; McIlvain *et al.*, 1994). The gathering of lysosomal granules may direct a "lethal hit" in the observed sequential fashion and target cells are destroyed without adjacent cells, not expressing the relevant antigens, being affected (Hahn *et al.*, 1994). CTL killing of target cells is characteristically unidirectional (Hahn *et al.*, 1994), however, the mechanisms which protect the CTL from its own cytotoxic mediators are not known. New data by Isaaz *et al.* (1995) suggests that synthesis of

granule contents occurs during target cell killing to re-fill the granules and that some of this newly synthesised material is released from the cell in a granule independent manner.

Experiments using perforin or Fas-knockout mice showed clearly that there are at least two separate cytolytic pathways used by CTLs; (i) a secretory and membranolytic delivery of a perforin based lethal hit and (ii) a non-secretory, Fas/APO-1 (CD95) receptor mediated triggering of target cell apoptosis (see Figure 7). Kagi *et al.* (1994a/1995) showed that perforin deficient mice were unable to recover from LCMV infection, whereas, Fas deficient mice recovered from LCMV infection similarly to normal mice. These experiments showed that CTLs were critical for the clearance of LCMV infection *in vivo* and that the perforin pathway was the mechanism of cytotoxicity used by these CTLs. The involvement of the Fas pathway in the clearance of virus infection by CTLs *in vivo* is still unknown, however, LCMV infected target cells could, in fact, be lysed by perforin deficient CTLs *in vitro* (Walsh *et al.*, 1994). The Fas pathway was attributed to this *in vitro* target cell killing because only Fas-bearing targets cells were destroyed by the perforin deficient CTLs (Kagi *et al.*, 1994b; Lowin *et al.*, 1994). It has been speculated that the two different mechanisms of CTL-mediated lysis may have evolved to protect against the multitude of escape strategies used by invading pathogens (Lowin *et al.*, 1994).

2.5.6 Secretory and membranolytic induced cell death

This mechanism involves Ca^{2+} dependent granule exocytosis and secretion of the pore-forming protein perforin along with a series of granule enzymes (granzymes) into the localised environment between the effector and target cell (Ishiura *et al.*, 1990; Peters *et al.*, 1991).

The lysosomal granules are unique organelles which contain a dense central core surrounded by a multivesicular cortex (Peters *et al.*, 1991). Components found in these granules include perforin, serine esterases (granzymes), cathepsin B and the calcium

FIGURE 7. TWO PATHWAYS OF CTL-MEDIATED TARGET CELL CYTOTOXICITY

CTLs recognise specific antigen in association with MHC class I (in green) on the surface of target cells via its TCR/CD3 complex (in grey). Signals are transmitted from the TCR/CD3 complex, along with co-stimulatory signals from other adhesion molecules, to activate the cytotoxic processes. There are believed to be two main pathways by which the cytotoxic effects of CTLs are mediated:

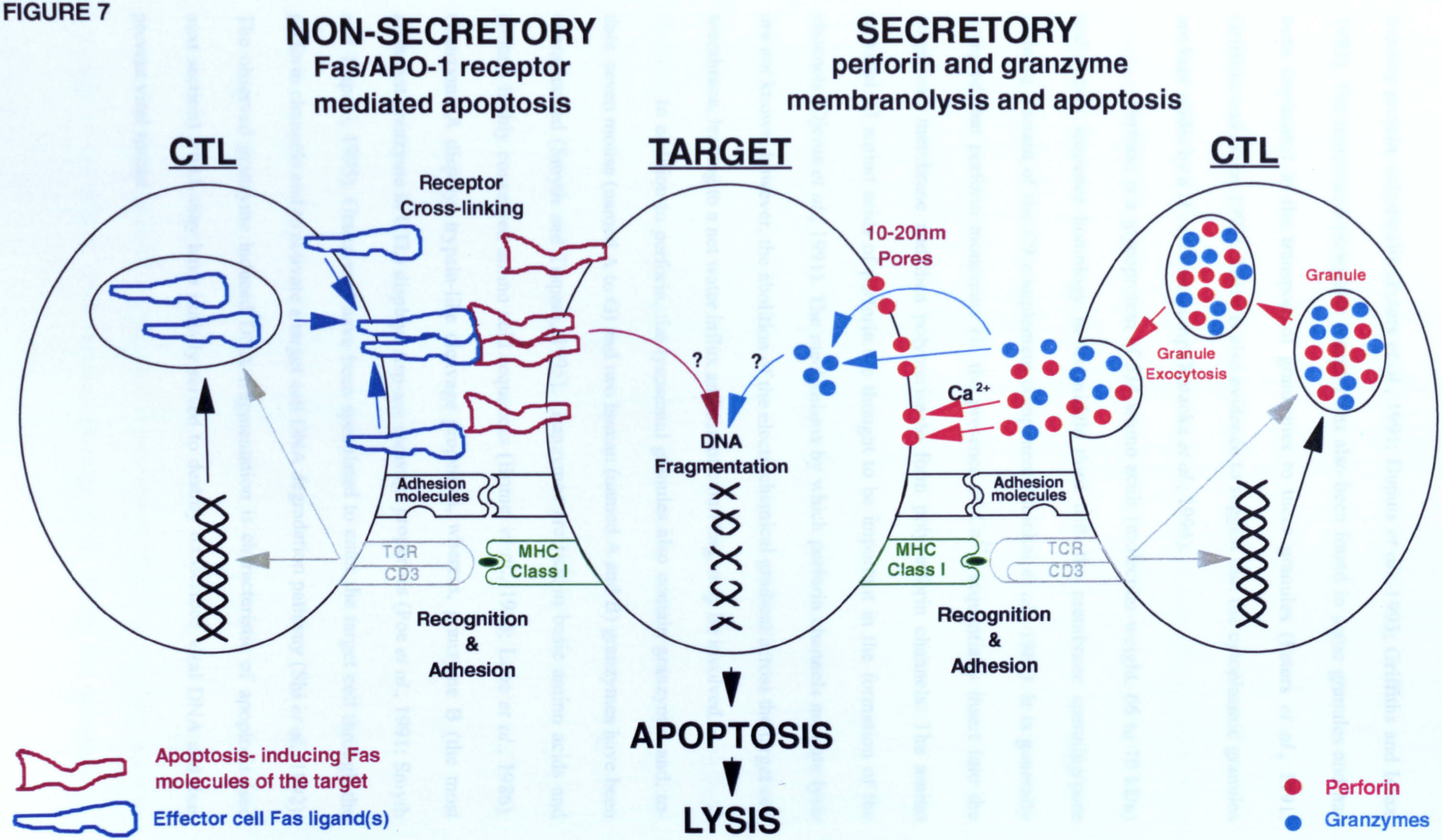
(i) Non-secretory cytotoxicity is believed to involve the *de novo* synthesis of Fas ligand (FasL) (in dark blue) and also the translocation of pre-made FasL to the CTL cell surface. Following interaction of the CTLs FasL and the Fas (in burgundy) on the target cell, the Fas molecules transmit a "death signal" which initiates target cell apoptosis.

(ii) Secretory cytotoxicity involves the translocation and secretion of cytoplasmic granules containing perforin (in red), granzymes A to G (in blue) and other cytotoxins. Many perforin proteins, in the presence of Ca^{2+}, bind to the target cell membrane and polymerise to form aqueous pores. Granzymes and other cytotoxins enter the target cell and transmit a "death signal" which initiates target cell apoptosis and lysis.

The biochemical signals involved in CTL induced apoptosis remain to be revealed.

Modified from Berke (1994) and Takayama (1995).

FIGURE 7


NON-SECRETORY
Fas/APO-1 receptor
mediated apoptosis
SECRETORY
perforin and granzyme
membranolysis and apoptosis
CTL
TARGET
CTL
Receptor
Cross-linking
10-20nm
Pores
Granule
Granule
Exocytosis
Ca 2+
?
?
DNA
Fragmentation
Adhesion
molecules
Adhesion
molecules
TCR
CD3
MHC
Class I
MHC
Class I
TCR
CD3
Recognition
&
Adhesion
Recognition
&
Adhesion
APOPTOSIS
LYSIS
Apoptosis- inducing Fas
molecules of the target
Effector cell Fas ligand(s)
Perforin
Granzymes

binding protein, calreticulin (Peters *et al.*, 1991; Dupuis *et al.*, 1993; Griffiths and Isaaz, 1993). The mannose-6-phosphate receptor has also been found in some granules and has been implicated in the transport of granzymes to these granules (Peters *et al.*, 1991; Griffiths and Isaaz, 1993). There is also evidence to suggest that the cytoplasmic granules are kept acidic by a H^+-ATPase pump (Kataoka *et al.*, 1994).

Perforin is a glycoprotein of 534 amino acids (molecular weight, 66 to 70 kDa) and shows sequence homology in its middle third with the membrane spanning/pore forming domain of the C9 complement component (Shinkai *et al.*, 1988). It is generally accepted that perforin monomers (in the presence of Ca^{2+}) sequentially insert into the target cell membrane and then polymerise to form polyperforin channels. The amino terminal 33 amino acids of perforin are thought to be important in the formation of the channels (Ojcius *et al.*, 1991). The mechanisms by which perforin channels mediate lysis are not known, however, the abolition of the electrochemical gradient across the target cell membrane, leading to a net water influx and osmotic bursting, may be involved.

In addition to perforin, the lysosomal granules also contain granzymes and, to-date, seven mouse (named A to G) and two human (named A and B) granzymes have been characterised (Smyth and Trapani, 1995). Granzymes are rich in basic amino acids and contain highly conserved amino acid sequences (Brunet *et al.*, 1986; Lobe *et al.*, 1986). Granzyme A displays trypsin-like cleavage properties, whereas, granzyme B (the most abundant granzyme in CTL) displays Asp-ase cleavage properties (Poe *et al.*, 1991; Smyth and Trapani, 1995). Granzymes have been speculated to enter the target cell through the perforin channels and to activate a target cell DNA degradation pathway (Shi *et al.*, 1992). The observed granzyme induced DNA fragmentation is characteristic of apoptosis (see next section) which may have initially served to destroy transcribed viral DNA and thus prevent viral spread.

2.5.7 Non-secretory and receptor-mediated induced cell death

Unlike perforin induced cell death which may result in membrane disruption and target cell necrosis, CTL-mediated target cell killing can also result in Ca^{2+} independent programmed cell death (apoptosis) via the Fas (mouse) or APO-1 (human) [CD95] receptor pathway (Rouvier *et al.*, 1993). Fas and APO-1 are cell surface glycoproteins (molecular weight, 35 kDa) which are found on both lymphoid and non-lymphoid tissues (Itoh *et al.*, 1991; Oehm *et al.*, 1992).

During CTL induced apoptosis, the target cell's DNA undergoes chromatin condensation and fragmentation (Russell, 1983). The target cell's plasma membrane undergoes blebbing which leads to cell fragmentation and the formation of sealed membrane fragments (apoptotic bodies) (Hahn *et al.*, 1994).

It has been suggested that following TCR and MHC-peptide binding, a signal is produced in the CTL via protein tyrosine kinase (PTK) to express the Fas ligand (FasL) (Anel *et al.*, 1994). FasL is presented on the CTL cell surface where it binds with Fas on the surface of the target cell (Suda *et al.*, 1993). This binding is believed to trigger second messengers including PTK which may affect the expression of the proto-oncogene Bcl-2 (Eischen *et al.*, 1994; Schroter *et al.*, 1995). The endogenous DNase I-like endonucleases are also thought to be involved in the target cell DNA fragmentation (Ucker *et al.*, 1992).

2.5.8 Non-cytolytic virus resolution

Although CTLs have been known to mediate a potent cytolytic activity *in vitro* for some time, the questions concerning the mechanisms of CTL activity both *in vitro* and *in vivo* have only recently been answered using the perforin-knockout mouse (see section 2.5.5). However, it should be noted that antiviral cytokines such as IFN-γ and TNF-α are produced by CTLs (and Th-lymphocytes) in response to viral infections and probably contribute to intracellular virus resolution (Ramsay *et al.*, 1993). For example, CTL

antiviral cytokines have been shown to non-cytolytically prevent mouse cells from expressing hepatitis B virus (HBV) genes *in vivo* (Guidotti *et al.*, 1994).

2.6 INTESTINAL INTRAEPITHELIAL LYMPHOCYTES

In addition to the circulating αβ TCR T-lymphocytes, there is a population of intestinal intraepithelial lymphocytes (iIELs) located between the epithelial cells lining the intestinal lumen. The iIELs are almost entirely T-lymphocytes which undergo thymus-independent development and selection within the intestinal epithelium (Guy-Grand *et al.*, 1978; Mosley *et al.*, 1990; Poussier *et al.*, 1992). The majority of mouse iIELs are $CD8^+$ T-lymphocytes, however, the TCR subset of these cells varies. The number of TCR-γδ cells appears to decrease, in parallel to the TCR-αβ subset increase, as the mouse ages (Maloy *et al.*, 1991; Takeuchi *et al.*, 1993). In humans, the majority of iIELs appear to be $CD8^+$ and TCR-αβ, although, up to thirty seven percent were TCR-γδ (Jarry *et al.*, 1990; Deusch *et al.*, 1991).

TCR-γδ lymphocytes appear to recognise the heat shock proteins, which are normally generated during the course of an infection, in an MHC-independent manner (Born *et al.*, 1990). TCR-γδ lymphocytes have also been found to interact with viral proteins in the absence of processing or MHC presentation (Sciammas *et al.*, 1994). For these reasons, it is thought that the TCR-γδ iIELs may act early in the immune response and provide a first line of defence against pathogens.

2.7 VIRUS-SPECIFIC MEMORY CTLs

The T-lymphocyte response to acute infection not only mediates viral clearance but provides the host with immunological memory, characterised by an increased frequency of memory T-lymphocytes (Th-lymphocytes and CTLs) responsive to antigenic epitopes expressed by the pathogen.

Influenza A virus infection of the mouse allows life-long recall of enhanced, virus-specific CTLs following *in vitro* stimulation (Effros and Walford, 1984). However, influenza A heterotypic protection (which is probably mediated by CTLs) appears to diminish *in vivo* at about five months after the primary infection (Liang *et al.*, 1994). In humans, memory CTLs to influenza A virus decline within two to three years after primary infection (McMichael *et al.*, 1983a). Also, humans vaccinated with variola virus (smallpox vaccine) have memory CTL to variolar virus in their peripheral blood for at least two to four years (Demkowicz and Ennis, 1993: Erickson and Walker, 1993).

From the current knowledge, it generally appears that naive CTL and memory CTL show contrasting cell surface markers; naive CTL display $CD45R^{hi}$, L-$selectin^{hi}$ and $CD44^{lo}$, whereas, memory CTL display $CD45R^{lo}$, L-$selectin^{lo}$ and $CD44^{hi}$ (Budd *et al.*, 1987; Lee and Vitetta, 1991; Vitetta *et al.*, 1991). Human memory CTLs appear to have a much more rapid turn-over than naive CTLs (Michie *et al.*, 1992), however, recent studies in the mouse have detected a proportion of memory CTLs (about twenty percent) which appear to be long lived and non-dividing (Tough and Sprent, 1994). Memory CTL generally circulate within the secondary lymphoid tissues (Razvi *et al.*, 1995).

It is not known how memory CTLs continue in circulation. Initial experiments showed that memory CTL responses to LCMV declined within days after the virus-specific CTLs were adoptively transferred to donor mice in the absence of antigen (Oehen *et al.*, 1992). These results suggested that memory CTLs may engage in continuous low-grade responses to residual depots of antigen. Follicular dendritic cells are known to sequester antigen on their surface for months or years (Tew *et al.*, 1990) and were speculated to maintain the survival of at least memory Th-lymphocytes (Sprent, 1994). More recently, however, LCMV-specific memory CTLs have been shown to survive for more than a year (Lau *et al.*, 1994) and Sendai virus-specific memory CTLs for more than 100 days (Hou *et al.*, 1994), following adoptive transfer into mice in the absence of

antigen. Hou *et al.* (1994) speculated that the primary clonal burst size may be the major influence in the longevity of memory CTL.

Akbar *et al.* (1993) suggested that the small percentage of T-lymphocytes which survived cell death, following the primary immune response to an antigen, were saved by accessory cells providing IL-2. IL-2 could switch on survival genes, including *bcl-2*, and allow the memory CTLs to survive in the absence of antigen.

Lastly, Selin *et al* (1994) have shown that memory CTLs specific for one virus could cross-react and contribute to the primary CTL response of an unrelated virus. Pichinde virus, vaccinia virus and MCMV all cross-reacted with LCMV-specific CTLs at the clonal level. Thus, memory CTLs may be constantly reactivated by remotely cross-reactive infectious agents and environmental antigens.

2.8 RESUME

The current view is that following virus infection at a peripheral site, professional antigen presenting cells (APCs) acquire, process and transport virus proteins to the lymphoid tissues. It is believed that viral proteins are degraded into peptides by the multi-catalytic protein protease (proteasome) in the cytoplasm of the professional APC and transported to the ER via the TAP transporter. In the ER, the peptides bind to MHC class I molecules and are then expressed on the cell surface. The highly polymorphic nature of the MHC proteins affects which viral peptides will be presented and, therefore, the nature of the CTL response. Precursor CTLs recognise, via their α/β TCR heterodimer, foreign (viral) peptide in association with MHC on the surface of the professional APC (MHC class I restriction) and become 'activated' effector CTLs.

Effector CTLs re-enter circulation and are directed to the peripheral site of virus infection. Virtually all somatic cells express MHC class I molecules and these amateur APCs are believed to present newly synthesised viral proteins in association with MHC

class I using the same pathway as the professional APCs. Effector CTL recognition of infected 'target' cells leads to target cell binding and then lysis, principally by the secretory (perforin) pathway.

Following resolution of the virus infection, it is believed that a small population of the effector CTLs survive and become memory CTLs. These cells are believed to circulate the host, respond rapidly to virus re-infection and protect the host from disease. Memory CTLs are particularly believed to be involved in the observed virus serotype-independent protection which can not be easily explained by the neutralising antibody response.

CHAPTER 3

THE IMMUNE RESPONSE TO ROTAVIRUS

3.1 INTRODUCTION

Despite the great deal of knowledge gained on the molecular biology of rotaviruses, the immunological mechanisms responsible for the clearance and subsequent protection from rotavirus infection still remains unclear. Indeed, the reason behind the mixed efficacy exhibited in the many vaccine trials that have been carried out is not known (reviewed in Conner *et al.*, 1994). Consequently, the relative importance of the antibody- versus the cell-mediated immune response to rotavirus infection is subject to much debate, especially in relation to the design of an effective vaccine. However, it should be noted that these two effector functions (and indeed other immunological mechanisms) may be inseparable.

The immune response following rotavirus infection has been studied most extensively in the mouse and, for this reason, the chapter will concentrate on the immune response in this animal model. It should be noted that in all the animals studied to-date, including humans and new-born calves, the mechanisms of the immune response appear to be fairly similar (Davidson *et al.*, 1983; Saif *et al.*, 1983; Sheridan *et al.*, 1983; Conner *et al.*, 1991).

3.2 COMPONENTS OF THE IMMUNE RESPONSE DURING ROTAVIRUS INFECTION

3.2.1 Location of rotavirus infection

Following oral inoculation of the suckling mouse with rotavirus, the virus replicates in the mature villus epithelial cells of the small intestine (Sheridan *et al.*, 1983). Although acute rotavirus disease is self-limiting, and the symptoms are normally resolved within a few days, the actual mechanisms of the immune response may depend on whether it is a primary infection or a secondary re-infection.

3.2.2 Immune response following primary infection

Following a primary rotavirus infection, professional antigen presenting cells (APCs), such as macrophages and dendritic cells, most probably acquire and process rotavirus antigens and transport them to the lymphoid tissues (Macatonia *et al.*, 1989; Hill *et al.*, 1990). Alternatively, infectious rotavirus (or viral antigen) might directly enter the blood and lymphatics, migrate to the spleen and mesenteric lymph nodes (MLN), and be taken up by local APCs (Eichelberger *et al.*, 1991). Regardless of the mode of travel, rotavirus antigens have been detected in the spleen by five days post infection (p.i.), and the MLN between three and twenty days p.i. (Dharakul *et al.*, 1988).

Within the lymphoid tissues, the professional APCs probably present rotavirus antigens to Th-lymphocytes, CTLs and antibody producing B-lymphocytes (London *et al.*, 1987; Hamilton-Easton and Eichelberger, 1995). The rotavirus-specific lymphocytes are likely to return to the site of infection following endothelium binding and extravasation (reviewed in Shimizu *et al.*, 1992). At this site, Th-lymphocytes probably release cytokines which promote B-lymphocyte antibody production and CTL activity.

3.2.3 Location and isotype of rotavirus-specific antibodies

Rotavirus-specific immunoglobulin (Ig)A secreting B-lymphocytes appear at the intestinal lamina propria four to six days p.i., peak in number at three weeks p.i., and remain for at least eight weeks (Merchant *et al.*, 1991). Although rotavirus-specific IgG secreting B-lymphocytes are also found in the lamina propria, albeit at a ten-fold lower number than IgA-secreting B-lymphocytes (Merchant *et al.*, 1991), the capacity of IgG to protect at the intestinal surface is not clear. The epithelial cell transcytosis of IgG cross-linked through multivalent antigen to polymeric IgA has been detected (Kaetzel *et al.*, 1994). IgG could, therefore, help mediate viral clearance through intracellular association with antigen and subsequent activation of complement.

Circulating B-lymphocytes also produce rotavirus-specific serum antibodies. Rotavirus-specific IgM appears in the serum three days p.i. followed by IgG and IgA (Sheridan *et al.*, 1983). Although it appears that only intestinal antibodies are directly involved in the clearance and protection from rotavirus infection (Offit and Clark, 1985a), it should be noted that dimeric serum IgA is removed from circulation by the liver and transported into the intestine via bile (Mestecky, 1987).

In humans, the subtype of IgA produced in response to rotavirus infection is largely IgA1. Older children and adults produce greater quantities of IgA2 which is more resistant to bacterial proteases and may correlate better with protection against rotavirus disease (Friedman *et al.*, 1996).

3.2.4 Location and TCR type of rotavirus-specific CTLs

Rotavirus-specific CTLs are detected in the intestinal intraepithelial lymphocytes (iIELs), Peyer's patch, MLN and spleen six days p.i. (Offit and Dudzik, 1989a) and are of the αβ $CD8^+$ subset (Offit *et al.*, 1991b).

3.2.5 Memory T- and B-lymphocytes are acquired following primary rotavirus infection

Following primary infection of mice with the murine rotavirus strain EDIM, the level of virus-specific faecal IgA decreased to ten percent of its peak within two months, but did not decrease further over the next twelve months. During this time the level of virus-specific serum IgA remained fairly constant and serum virus-specific IgG steadily increased (McNeal and Ward, 1995). Similarly in humans, the rise in faecal rotavirus-specific IgA is frequently transient (Coulson *et al.*, 1992; Matson *et al.*, 1993), whereas, serum IgA levels persist for at least four months (Grimwood *et al.*, 1988).

Although it is not known how long rotavirus-specific memory CTLs persist following rotavirus infection, this question has been addressed in other virus systems. It appears that influenza virus-specific CTLs remain in the mouse for the remainder of its life (Effros and Walford, 1984), however, protection from influenza re-challenge diminishes at about five months after the primary infection (Liang *et al.*, 1994). Also in humans, memory CTLs specific for influenza or variola virus exist for approximately three years in the absence of further challenge (McMichael *et al.*, 1983a; Demkowicz and Ennis, 1993; Erickson and Walker, 1993).

3.2.6 Immune response following re-infection

Following rotavirus re-infection, it is likely that amateur APC (probably including B-lymphocytes) acquire and process rotavirus antigens and present them to rotavirus-specific memory lymphocytes within the local site of infection, Peyer's patches and lamina propria (Macatonia *et al.*, 1989; Fuchs and Matzinger, 1992; Azuma *et al.*, 1993).

3.3 BOTH ANTIBODY AND CTLs ARE INVOLVED IN CLEARANCE OF THE PRIMARY ROTAVIRUS INFECTION

A number of studies have indicated that both the cell-mediated and humoral immune response mechanisms are required for the proficient clearance of a primary rotavirus infection.

Firstly, chronic rotavirus infections of SCID mice were ablated by the passive transfer of CTLs from MHC class I matched Balb/c mice previously inoculated with infectious rotavirus (or single rotavirus proteins expressed by recombinant baculoviruses). However, rotavirus shedding recurred at a low level which indicated that either, (i) the CTLs were generating a selection pressure and this selection pressure was generating a variant viral population, or (ii) that CTLs alone were not sufficient in clearing chronic

rotavirus infections (Dharakul *et al.*, 1990/1991). Very recently, J_HD-knockout mice (B-lymphocyte-deficient) were found to clear primary murine rotavirus infection as would normal mice, suggesting that CTLs are involved in the clearance of rotavirus infection. The same experiments were carried out in μMT-knockout mice (B-lymphocyte-deficient) and, although the initial infection was cleared, these mice suffered from subsequent bursts of renewed virus shedding. Depleting the CTLs from both these two mice strains either ablated rotavirus clearance (J_HD-knockout mice) or prolonged virus clearance (μMT-knockout mice) and confirmed that CTLs were involved in the resolution of primary rotavirus infection (McNeal *et al.*, 1995).

Secondly, β_2m-knockout mice (CTL-deficient) cleared primary rotavirus infection at a delayed rate (Franco and Greenberg, 1995) and increased viral release was also seen in calves treated with an anti-CD8 monoclonal antibodies (Oldham *et al.*, 1993).

Thus, taking these experiments together, it appears that both T- and B-lymphocytes participate in the resolution of the primary rotavirus infection (Franco and Greenberg, 1995; McNeal *et al.*, 1995).

3.4 NEITHER ANTIBODY NOR CTLs ARE INVOLVED IN THE CLEARANCE OF THE PRIMARY ROTAVIRUS INFECTION

T-lymphocyte-deficient (nude) mice have been found to clear rotavirus infection in the absence of a significant antibody response or a functional T-lymphocyte response (Eiden *et al.*, 1986). Also, the resolution of rotavirus in J_HD and μMD-knockout mice (B-lymphocyte-deficient), depleted of $CD8^+$ CTLs, showed that other effector functions may also have a role in the clearance of rotavirus infection (McNeal *et al.*, 1995). These include both immunological mechanisms such as $CD4^+$ CTLs, natural killer (NK) cells, and non-immunological mechanisms such as the depletion of susceptible villus epithelial cells. However, although these mice cleared the primary infection, the mice were not actually

protected from re-infection. This strongly suggests that either memory T- and/or B-lymphocytes are required for subsequent protection (McNeal *et al.*, 1995).

3.5 GENERAL PROPERTIES OF PROTECTION FROM ROTAVIRUS RE-INFECTION

3.5.1 Primary rotavirus infection protects the host from subsequent re-infection

There are numerous examples of both humans and animals being protected from rotavirus re-infection following primary infection. Mice orally inoculated with EDIM were protected against re-infection with the same virus (Ward *et al.*, 1990c) and this protection remained for at least fourteen months (most of the lifetime of the mouse) (McNeal and Ward, 1995). Protection from subsequent episodes of serious rotavirus disease has also been observed in infants following the recovery from either symptomatic or asymptomatic natural infections (Bishop *et al.*, 1983; Bernstein *et al.*, 1991; Coulson *et al.*, 1992; Ward and Bernstein, 1994).

The role of the immune system in amelioration of primary infection and protection from re-infection and/or disease is supported by the fact that severe combined immunodeficiency (SCID) mice become chronically infected with rotavirus (Riepenhoff-Talty *et al.*, 1987) as do immunodeficient children (Hundley *et al.*, 1987).

3.5.2 Heterotypic protection is generated following rotavirus infection

The hosts immunity to rotavirus not only protects against re-infection and/or disease by rotavirus of the same VP7 (G) and VP4 (P) serotype (homotypic protection/serotype-dependent) but may also protect against rotaviruses of different G and P serotypes (heterotypic protection/serotype-independent). There are, however, conflicting reports as to whether this heterotypic protection develops following primary infection (Bridger and

Oldham, 1987; Woode *et al.*, 1987) or following two or more infections (Snodgrass *et al.*, 1991; Brussow *et al.*, 1991a/b).

3.5.3 Protection may correlate to the magnitude of the primary immune response

As mentioned in sections 3.5.1 and 3.5.2, natural infection with rotavirus can offer protection to subsequent infection and/or disease. However, protection following rotavirus infection has also been seen to be limited in infants and to not prevent multiple rotavirus illnesses (Friedman *et al.*, 1988; Coulson *et al.*, 1992) or multiple asymptomatic infections (Matson *et al.*, 1993).

One possible reason for the variability in protection following rotavirus infection was revealed in vaccine trials. Candidate vaccine strains for infants based on heterologous rotaviruses, which are known to replicate poorly in humans, have provided only partial protection (Berstein *et al.*, 1990; Santosham *et al.*, 1991). A similar situation occurs in the mouse model in which the murine rotavirus EDIM protected mice from subsequent EDIM infection whereas the heterologous RRV virus failed to provide protection from subsequent EDIM infection (Ward *et al.*, 1992). Using rotavirus EDIM/RRV reassortants, no individual EDIM protein was found to correlate with protection, whereas, rotavirus replication (measured by levels of both rotavirus shedding in stools, and serum IgA) was found to correlate with protection (McNeal *et al.*, 1994). Although the level of rotavirus replication is an attractive hypothesis for the variability seen in rotavirus protection, it does not account for the fact that parenterally inoculated, non-replicating, rotaviruses also offer protection to re-infection via the natural route, as described in the next section.

3.5.4 Rotavirus replication is not required at the intestinal surface to elicit an immune response

Although rotaviruses normally enter the host by the faecal-oral route and replicate in the epithelial cells of the small intestine, rotavirus replication or diarrhoea is not actually required for the generation of protection. For example, rotavirus-specific T- and B-lymphocytes develop following: (i) oral inoculation of the adult mouse, which does not support rotavirus replication, and (ii) parental inoculation of both suckling and adult mice, where virus does not encounter the intestine (Offit and Dudzik, 1989a/b; Conner *et al.*, 1993). The route of rotavirus inoculation does, however, alter the antibody response. For example, the parenteral inoculation of mice leads to an increase in rotavirus-specific intestinal IgG, and to a decrease in intestinal rotavirus-specific IgA which is associated with infection by the natural route (Coffin *et al.*, 1995).

3.6 THE ROLE OF THE HUMORAL IMMUNE RESPONSE IN PROTECTION FROM ROTAVIRUS RE-INFECTION

3.6.1 Antibodies are involved in protection

As described in section 3.2.3, clearance of rotavirus infection by the host is accompanied by the appearance of rotavirus-specific antibodies and the levels of these antibodies have been correlated with protection from subsequent re-infection. For example, active immunity against rotavirus infection has been correlated with pre-existing levels of serum and faecal rotavirus-specific IgA (but not serum IgG) in the mouse model (Feng *et al.*, 1994; McNeal *et al.*, 1994; Burns *et al.*, 1995). Also, serum rotavirus-specific IgA and IgG levels were correlated with protection against rotavirus infection and illness of infants in an orphanage (O'Ryan *et al.*, 1994). In addition, faecal IgA alone was correlated with protection of children in hospitals and day care centres (Coulson *et al.*, 1992; Matson *et al.*, 1993).

More direct evidence of a role for antibodies in protection from rotavirus was shown when milk from rotavirus inoculated dams, administered orally to suckling mice, protected the suckling mice from subsequent disease following challenge (Offit and Clark, 1985a; Offit and Dudzik, 1989b). Although rotavirus-specific milk IgA was found to be tenfold more potent than milk IgG, both could independently passively protect against disease (Offit and Clark, 1985a).

3.6.2 Serotype-dependent antibodies develop after primary infection, whereas, serotype-independent antibodies develop after re-infection

It is generally accepted that the primary exposure to rotavirus produces a serotype-dependent neutralising antibody response against the serotype of the infecting strain and that the serotype-independent antibody response develops with subsequent infections. For instance, children with high G serotype 3 serum antibodies (but not those with high G serotype 1, 2 or 4, but low 3, serum antibodies) were protected from disease following serotype 3 challenge (Chiba *et al.*, 1986). Re-infections of children were found to boost antibody levels to more than one G serotype (Coulson *et al.*, 1992) and adults elicited a broad heterotypic antibody response following rotavirus infection (Ward *et al.*, 1989).

There are two hypotheses concerning the generation of serotype-independent antibodies. The older view is that re-infections with different rotavirus strains boost neutralising antibodies against the different serotypes to which the host has previously been exposed. However, the re-infections do not broaden the response to include serotypes to which the host has not previously encountered (Offit and Clark, 1985b; Snodgrass *et al.*, 1984; Zheng *et al.*, 1988).

More recently, studies in both calves and children have revealed that the serotype-independent antibody response is generated without previous exposure to those serotypes (Snodgrass *et al.*, 1991; Brussow *et al.*, 1991a/b). This has lead to the new

hypothesis that a broad serotype-independent antibody response develops after exposure to only a few different serotypes because of the presence of minor cross-neutralising epitopes (see next section).

3.6.3 Minor cross-neutralising epitopes may be involved in heterotypic protection

The serotype-independent antibody response may result from the existence of minor cross-neutralising antibody epitopes which are masked by immunodominant neutralisation epitopes during primary infection. It is speculated that if the primary and secondary infections are very similar, then the bulk of the antibody response will be directed to the major neutralisation antigens of the second virus. If, however, the primary and secondary rotavirus infections are sufficiently different, then the bulk of the antibody response will be directed, via memory B-lymphocytes, to the minor serotype-independent epitopes. This process (named antigenic sin) will also direct the antibody response toward the primary rotavirus epitopes (Snodgrass *et al.*, 1991).

A good example of this was seen when administrating the WC3 bovine vaccine strain (serotype 6) after a natural rotavirus infection (serotype 1) (Ward *et al.*, 1990d). Primary infection with the high replicating natural rotavirus strain elicited a heterotypic response to serotype 1 (the infecting strain) followed by serotype 3, and low response against serotype 4 and 2. Following WC3 re-infection, the neutralising antibody responses to all these serotypes were boosted (approximately twelve-fold) in proportion to each other.

3.6.4 Antibodies recognise most rotavirus proteins

Human sera obtained during the convalescent phase following rotavirus infection contained IgG antibodies directed against inner capsid proteins VP1, VP2, VP3 and VP6, outer capsid proteins VP4 and VP7, and non-structural proteins NS53, NS34 and NS35.

The non-neutralising VP2 and VP6 rotavirus proteins were immunodominant (Svensson *et al.*, 1987a/b). This polyclonal antibody response to various rotavirus proteins appears to be mirrored by the serum IgA1 antibody response (Johansen *et al.*, 1994). It is worth noting that both the IgG and IgA1 antibody responses to the neutralising VP4 and VP7 proteins were low compared to the other rotavirus proteins.

3.6.5 VP4 and VP7 both contain major neutralising, and minor cross-neutralising, antibody epitopes

The external virion shell proteins VP4 and VP7 produce neutralising antibodies and, therefore, form the basis of rotavirus serotyping. Consequently, the protective antibody response to these two proteins has been studied extensively. Offit *et al.* (1986a) showed, using VP4 and VP7 reassortant rotaviruses, that both these proteins could independently evoke virus-neutralising antibodies in the milk of inoculated dams which passively protected suckling mice against diarrhoea. This passive protection was also generated using monoclonal antibodies directed against either VP4 or VP7 (Offit *et al.*, 1986b; Matsui *et al.*, 1989).

Having confirmed that VP4 and VP7 could separately evoke a protective antibody response, disagreement then arose as to which of the these two proteins was immunodominant. VP4 and VP7 reassortant rotavirus experiments in humans showed, in some cases, that approximately ninety-five percent of the neutralising antibody response was directed against VP4 (Ward *et al.*, 1988; Flores *et al.*, 1989). The immunodominance of VP4 over VP7 has also been detected following natural infections of infants (Offit *et al.*, 1993; Svensson *et al.*, 1987a; Ward *et al.*, 1993). However, other VP4 and VP7 reassortant rotavirus experiments showed that the majority of the neutralising antibody was directed at VP7 (Ward *et al.*, 1990b). Possible reasons for the conflicting results include, (i) a changing immunodominant protein which depends on whether the antibodies

were generated following primary or secondary infection, or (ii) experiments involving reassortants may give unpredictable results associated with the fact that the antigenicity of VP7 depends on the VP4 background, and vice versa (Chen *et al.*, 1989/1992; Dunn *et al.*, 1994).

The earlier experiments by Offit *et al.* (1986b) and Matsui *et al.* (1989) also showed that monoclonal antibodies directed against either VP4 or VP7 passively protected suckling mice against diarrhoea induced by different rotavirus serotypes. These results suggested that both VP4 and VP7 contained serotype-independent epitopes. More recently, experiments by Taniguchi *et al.* (1991) showed that the polyclonal antibody response generated following the re-infection of children with rotavirus included neutralising antibodies directed at minor, serotype-independent, epitopes on both VP4 and VP7. The authors speculated that this response probably followed the principles of antigenic sin as described in section 3.6.3.

The locations of the VP4 and VP7 major neutralising, and minor cross-neutralising, antibody epitopes have been described in sections 1.7.2 and 1.7.3.

3.6.6 A role for non-neutralising VP6 antibodies in heterotypic protection

Recently, a functional role for non-neutralising serum IgA antibody was found by implanting mice with hybridoma cells secreting VP6-specific IgA antibody (Burns *et al.*, 1996). VP6-specific serum IgA (but not passively fed VP6-specific IgA) protected the mice from infection with both homotypic and heterotypic rotavirus strains and, indeed, VP6 is antigenically conserved among different mammalian Group A rotaviruses (Gorziglia *et al.*, 1988). VP6 has also been shown to contain rotavirus strain-independent Th-lymphocyte epitope(s) (Bruce *et al.*, 1994) and is highly immunodominant for both the IgG and IgA antibody responses in infants (Svensson *et al.*, 1987a; Johansen *et al.*, 1994).

The locations of the VP6 non-neutralising, antibody epitopes have been described in section 1.7.1.

3.6.7 Antibodies may not be directly involved in protection

Antibody responses are only one of the mechanisms employed by the immune system to clear virus infection. In fact, other mechanisms may be involved in primary rotavirus clearance and protection from re-infection or disease, and antibody may only serve as an indicator of their presence.

There are a number of reasons why the role of the antibody response in clearance and protection from rotavirus infection is questioned. Firstly, Ward *et al.* (1992) found that certain rotavirus strains protected mice from EDIM re-infection even though EDIM-specific neutralising antibodies in the intestine were not detectable at the time of re-inoculation. Also in mice, correlations between protection and titres of serotype-specific antibodies in the serum or faeces were not observed (McNeal *et al.*, 1992).

Gnotobiotic calves inoculated with either avirulent or virulent rotavirus strains of one serotype were protected against a virulent rotavirus strain of a different serotype in the absence of rotavirus-specific neutralising antibodies in either the serum or faeces to the challenge strain (Bridger and Oldham, 1987; Woode *et al.*, 1987).

Finally, infants immunised with the WC3 or RIT 4237 vaccines, which are serotypically distinct from human strains, were protected from disease caused by serotype 1 human rotavirus despite the absence of virus-specific antibodies in their sera (Vesikari *et al.*, 1985; Clark *et al.*, 1988). Moreover, Ward *et al.* (1990a) predicted, from serum rotavirus-specific IgG titre, that thirteen out of twenty adults would have been infected after homotypic re-challenge with the CJN strain. In fact only three subjects were infected upon re-challenge which indicated that a second effector arm of the immune system may be associated with protection against rotavirus re-infection.

3.7 THE ROLE OF THE CELL-MEDIATED IMMUNE RESPONSE IN PROTECTION FROM ROTAVIRUS DISEASE

3.7.1 CTLs are involved in protection

CTLs, in the absence of rotavirus-specific antibody, have been shown to clear rotavirus infection and prevent disease. Firstly, suckling mice intraperitoneally inoculated with splenic lymphocytes from adult C57BL/6 (H-2^b) or Balb/c (H-2^d) mice inoculated with murine rotavirus, provided them with protection against disease caused by murine rotavirus challenge (Offit and Dudzik, 1990). Further, SCID mice orally administered with CTLs from adult Balb/c mice, previously inoculated intraperitoneally with murine rotavirus, ablated a chronic murine rotavirus infection (Dharakul *et al.*, 1990).

RRV rotavirus-specific CTLs generated in C57BL/6 mice were able to lyse H-$2D^b$, but not H-$2K^b$, restricted target cells infected with RRV rotavirus within two hours *in vitro* (Offit *et al.*, 1989). The production of infectious RRV rotavirus takes between four and eight hours in these target cells and this may have important consequences during natural rotavirus infections. Indeed, a logarithmic phase of rotaviral growth involving multiple cycles of viral replication usually occurs 36 to 48 hours after infection in mice (Starkey *et al.*, 1986). Effector CTLs are generated from CTL precursors within 48 hours after infection and could therefore lyse virus infected cells prior to the logarithmic phase of infectious virus production (Offit *et al.*, 1989).

3.7.2 The involvement of CTLs in heterotypic protection

The ability of rotavirus-specific CTLs generated to one rotavirus serotype to kill target cells infected with rotavirus of another serotype has been described. For example, CTLs generated from C57BL/6 (H-2^b) mice inoculated with either bovine, simian or human rotaviruses could lyse target cells infected with rotaviruses of different serotypes (Offit

and Dudzik, 1988). In fact, Offit and Svoboda (1989) showed that the protection of infants against serotype 1 rotavirus provided by the serotypically distinct WC3 vaccine, may be explained by the CTL response. Mice orally inoculated with WC3 developed CTLs which could lyse target cells infected with heterotypic serotype 1 rotavirus. Lastly, the passive transfer of CTLs generated from adult mice, previously inoculated with one of many different rotavirus serotypes, could ablate a chronic murine rotavirus infection of a different serotype in SCID mice (Dharakul *et al.*, 1991).

3.7.3 CTLs recognise many rotavirus proteins

In C57BL/6 (H-2^b) mice inoculated with UKtc, SA11 or RRV, a rotavirus-specific CTL response was directed against target cells expressing VP7. In addition, CTLs from mice inoculated with UKtc or SA11 weakly lysed target cells expressing UKtc VP4 or SA11 VP6, respectively (Offit *et al.*, 1991a). Further studies found rotavirus VP7, but not VP1, VP2, VP4, NS53, VP6, NS35, NS28 or NS26, -specific CTLs were generated in C57BL/6 mice inoculated with recombinant vaccinia viruses expressing the individual SA11 proteins (Offit *et al.*, 1994). Thus, using the recombinant vaccinia virus system to individually express nine (of the twelve) rotavirus proteins, there only appeared to be a response to the VP7 protein in C57BL/6 mice. The vaccinia expression system has also been used to express individual rotavirus RF proteins for use in CTL assays. In this case, as well as VP7, a rotavirus-specific CTL response was again detected against VP6, but not VP1, VP2 or VP3, when rotavirus RF was used to inoculate C57BL/6 mice (Franco *et al.*, 1994).

Individual rotavirus proteins expressed by recombinant baculoviruses were also used to inoculate mice instead of infectious rotavirus. Earlier experiments showed that transferred rotavirus-specific CTLs, which mediated the clearance of chronic rotavirus infection from SCID mice, could be independently generated by inoculating Balb/c (H-2^d)

mice with baculovirus individually expressed VP1, VP4, VP6 or VP7, but not VP2, NS53, NS35 or NS28 rotavirus proteins (Dharakul *et al.*, 1991). Then, using RF recombinant baculovirus proteins to inoculate C57BL/6 mice, rotavirus-specific CTL responses were found against target cells expressing rotavirus VP3 and VP6 proteins, but not targets expressing VP1 (Franco *et al.*, 1994). Although these two experiments cannot be directly compared because of the use of different mouse strains and different rotavirus proteins, combining the results suggests that CTL epitopes are found on VP2, VP3, VP4, VP6, VP7 and, with disagreement, VP1.

3.7.4 The MHC class I haplotype affects the CTL response

The rotavirus epitopes presented to CTLs depends on the particular MHC class I haplotype. To date, rotavirus-specific CTL epitopes in haplotypes of mouse other than H-2^b have only been detected using the baculovirus expressed proteins to inoculate the mice. A rotavirus-specific CTL response directed to VP1, VP4, VP6 and VP7 has been detected in Balb/c (H-2^d) mice (Dharakul *et al.*, 1991), however, using VP1, VP2, VP3 and VP6 proteins, Franco *et al.* (1994) found no RF-specific CTL response in Balb/c mice and only a VP3-specific CTL epitope in C3H/He,mg (H-2^k) mice.

3.7.5 Serotype-independent VP7-specific CTL epitopes have been detected

Serotype-independent, rotavirus-specific CTLs generated in C57BL/6 mice after oral inoculation with rotavirus SA11 or RRV (both serotype 3), or UKtc (serotype 6), cross-lysed target cells expressing VP7 of serotypes 3 or 6 (Offit *et al.*, 1991a). This was confirmed by Offit *et al.* (1994) when CTLs generated from the inoculation of mice with recombinant vaccinia viruses expressing rotavirus SA11 or UKtc VP7 lysed target cells infected with rotavirus serotypes 3 or 6. To-date, no other rotavirus proteins have been found to contain cross-specific CTL epitopes.

3.7.6 Location of epitopes recognised by CTLs

Using allele-specific motifs to predict MHC class I mouse H-2^b epitopes, a synthesised peptide covering amino acids 33 to 40 of the rotavirus RF VP7 protein (which overlaps the H2 signal peptide located between amino acids 33 to 48) was recognised by rotavirus-specific CTLs. This epitope was found to be restricted at H-$2K^b$ (Franco *et al.*, 1993) and, in addition, the region of the VP7 protein containing this epitope is conserved, although not completely conserved, between different rotavirus strains (Estes and Cohen, 1989). In the light of this, and the fact that there is known to be a serotype-independent CTL response against VP7, the view emerged that this region defined that serotype-independent epitope. However, no experimental evidence showing a serotype-independent CTL response to this epitope has been reported.

A VP7-specific CTL response in other haplotypes of mice has only been detected using the baculovirus expressed rotavirus RRV protein to inoculate Balb/c (H-2^d) mice. The location of the epitope(s) recognised by VP7-specific CTLs generated in Balb/c mice have yet to be determined (Dharakul *et al.*, 1991).

Using baculovirus expressed individual rotavirus RF proteins to inoculate C57BL/6 mice, VP3 and VP6 were found to contain CTL epitopes. Using allele-specific motifs to predict MHC class I mouse H-2^b epitopes, peptides synthesised for VP3 (amino acids 585 to 593) and VP6 (amino acids 376 to 384) were recognised by rotavirus-specific CTLs; furthermore, both of these epitopes were restricted at H-$2K^b$. Initial experiments have revealed that rotavirus RF VP3 contains epitopes recognised by CTLs generated in C3H/He,mg (H-2^k) mouse, but, the location of the rotavirus-specific H-2^k CTL epitope(s) in this protein have yet to be determined (Franco *et al.*, 1994).

Although various experiments using baculovirus expressed individual rotavirus proteins to inoculate mice have also identified a CTL response to other rotavirus proteins,

VP1, VP2 and VP4 (Dharakul *et al.*, 1991; Franco *et al.*, 1994), the location of the rotavirus-specific CTL epitope(s) in these proteins have yet to be determined.

3.7.7 CTLs may not be directly involved in protection

Very recent work showed that both J_HD and μMT-knockout mice (B-lymphocyte-deficient), which had cleared a primary infection, were not protected from murine rotavirus re-infection, whereas, β_2m-knockout mice (CTL-deficient) were completely protected and this protection correlated with the development of a rotavirus-specific intestinal IgA response. Thus antibodies, not CTLs, appear to protect against rotavirus re-infection in these mice (Franco and Greenberg, 1995; McNeal *et al.*, 1995).

It should be noted that the genetically engineered mice used in these experiments have limitations due to the functional redundancy of the immune system. The inability of B-lymphocyte-deficient mice to resolve rotavirus re-infection can be explained in various ways: (i) following secondary rotavirus infection, it is probable that amateur APCs (including B-lymphocytes) present rotavirus antigens to Th-lymphocytes and CTLs within the local site of infection (Macatonia *et al.*, 1989; Fuchs and Matzinger, 1992; Azuma *et al.*, 1993). The presence of B-lymphocytes could, therefore, be essential for the direct stimulation of rotavirus-specific memory CTLs. Alternatively, the B-lymphocytes could present antigenic epitopes to Th-lymphocytes which then provide the cytokines necessary for memory CTL effector functions, (ii) memory CTL activity *in vivo* could, in part, be directed to rotavirus infected cells via antibody-dependent cell-mediated cytotoxicity (ADCC) or (iii) CTLs have the capacity to protect against disease, but not against the viral replication as measured in these studies.

The ability of CTL-deficient mice to prevent rotavirus replication (as measured by viral shedding) only suggests that CTLs are not essential, nevertheless, CTLs may still be involved when present. For instance, heterotypic protection can not easily be explained

by the neutralising antibody response and, therefore, it would be interesting to see if CTL-deficient mice are also protected from heterotypic rotavirus re-infection.

3.8 IMPLICATIONS FOR ROTAVIRUS VACCINE DESIGN

Studies on the immunity to rotavirus infection have highlighted three crucial parameters; (i) the immune response clears the primary infection, (ii) the immune response to the primary infection provides subsequent protection to rotavirus re-infection and/or disease, and (iii) the protection from re-infection and/or disease is serotype-independent (heterotypic protection). In principle, a rotavirus vaccine administered prior to the first exposure (at two to six months of age), which is capable of inducing protective immunity to all the prevalent circulating serotypes, could be developed.

Several vaccine trials using live, oral, attenuated (heterologous animal) viruses "traditional vaccine", or genetic reassortant viruses containing genes encoding VP7 and/or VP4 of selected human rotavirus strains in an animal virus background "modified traditional vaccine", have been undertaken. Unfortunately, the large field trials with these vaccines have revealed mixed efficacy, regardless of the development of neutralising antibodies (reviewed in Conner *et al.*, 1994).

An antibody-promoting vaccine containing all the serotypes to which the host may encounter could be effective, but this type of vaccine will be difficult to construct and probably need to be updated frequently. In addition, single serotype-specific antibody-promoting vaccines will probably generate a broad immune response after two or more inoculations with different serotypes. This raises problems in that multiple doses could be difficult and expensive to administer, especially in many areas of the developing world.

A CTL-promoting vaccine, on the contrary, has the potential to cross-protect against disease caused by many of the different rotavirus serotypes following a single dose, presenting a cheap vaccine in terms of both production and administration. However,

in contrast to the antibody response, the understanding of the rotavirus-specific CTL response is still in its infancy. An important step in the development of a CTL-promoting vaccine would be to identify all the rotavirus protein(s) which are involved in stimulating a CTL response, particularly if the CTL response generated by those proteins were serotype-independent.

CHAPTER 4

MATERIALS AND METHODS

4.1 MATERIALS

4.1.1 Standard buffers and solutions

The following standard buffers and solutions were used throughout this study:

10X loading buffer: 20% Ficoll 400, 0.1M ethylenediamine tetracetic acid (EDTA) pH 8.0, 1.0% sodium dodecyl sulphate (SDS), 0.25% bromophenol blue and 0.25% xylene cyanol

TAE: 40 mM Tris. Acetate and 2 mM EDTA pH 8.0

TBE: 89 mM Tris base, 89 mM boric acid and 2 mM EDTA pH 8.0

Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$ and 1.4 mM KH_2PO_4

2X polymerase chain reaction (PCR) mixture: 50 µl 10X Reaction Buffer-Mg^{2+} free (0.5M KCl, 0.1M Tris-HCl pH 9.0, 1% Triton X-100), 50µl 25 mM Mg^{2+}, 4µl 5 mM dNTPs, 10U Taq polymerase and distilled water to 250 µl total volume

2X reverse transcriptase (RT)-PCR reaction mixture: as 2X PCR reaction mixture including 5U reverse transcriptase

4.1.2 Tissue culture media and cell lines

Sterile PBS, Versene (0.02% in PBS), neutral red (0.1% in Earles saline), L-glutamine, penicillin and streptomycin sulphate [100,000,000U/100g in 200 ml PBS], trypsin (0.25% in PBS), 2X (2.8%) Noble Agar and tissue culture media including: Dulbecco's modification of Eagle's minimal essential medium (DMEM), Glasgow modification of Eagle's minimal essential medium (GMEM) plus non-essential amino acids (NEAA; 0.89g L-alanine, 1.15g L-asparagine.H_20, 1.33g L-aspartic acid, 0.75g glycine, 1.45g L-glutamic acid, 1.15g L-proline and 1.05g L-serine per litre) 1X, 2X and Roswell Park Memorial Institute (RPMI) 1640 plus Hepes and $NaHCO_3$, were all prepared by the media preparation staff of the Department of Biological Sciences, University of Warwick.

Table 3. Description of cell lines and their culture media

Cell Type	Description	Culture Medium
BS-C-1	African Green Monkey Kidney (ATCC CCL 26)	GMEM (5% FCS)
CV-1	African Green Monkey Kidney (ATCC CCL 70)	GMEM (5% FCS)
EL4	C57BL/6N mouse lymphoma (ATCC TIB 39)	RPMI (10% FCS)
HuTK$^-$ 143B	Human osteosarcoma Thymidine kinase negative 5-bromo-2-deoxyuridine resistant (ATCC CCL 8303)	GMEM (5% FCS)
Ltk$^-$	Mouse connective tissue Thymidine kinase negative (Supplied by Dr. Quinn, University of North Carolina)	DMEM (10% FCS)
L-D^b	Mouse connective tissue Restricted at D^b Resistant to HAT (Supplied by Dr. D. Quinn, University of North Carolina)	DMEM (10% FCS)
L-K^b	Mouse connective tissue Restricted at K^b Resistant to G418 (Supplied by Dr. D. Quinn, University of North Carolina)	DMEM (10% FCS)
P815	DBA/2 mouse mastocytoma (ATCC TIB 64)	RPMI (10% FCS)

ATCC® is the registered trademark of the American Type Culture Collection.

Table 4. Contents of growth media

1X Medium	FCS/ Myoclone FCS	L- glutamine	β-mercapto-ethanol	Penicillin	Streptomycin
DMEM	10% FCS	2 mM	-	100 U/ml	100 µg/ml
GMEM	5% FCS	2 mM	-	100 U/ml	100 µg/ml
RPMI	10% FCS	2 mM	-	100 U/ml	100 µg/ml
RPMI DM	5%, 10% Myoclone FCS	2 mM	50 µM	100 U/ml	100 µg/ml

Dutch modified RPMI 1640 medium (RPMI DM), Foetal Calf Serum (FCS) and Myoclone super plus FCS were purchased from Gibco/BRL. FCS was routinely heat treated at 56°C for 1 hr before use. Table 3 shows the cell lines used along with their corresponding media, while Table 4 shows the contents of routinely used media.

4.1.3 Primers

The oligonucleotide primers utilised during this study are shown in Table 5. Primers were synthesised by L. Ward, Department of Biological Sciences, University of Warwick using the Applied Biosystems 380B synthesiser, according to the manufacturer's instructions.

4.1.4 Vectors

The vectors used in this project were; (i) pCR™ II from Invitrogen's TA Cloning™ system, (ii) pGS62 (Smith *et al.*, 1987), (iii) rotavirus genes 2 and 3 of strain UKtc, gene 5 of strains RRV, Hochi and P9DΔ5 and the gene (7, 8 or 9) encoding the VP7 protein of strains RRV, A64, Hochi, 69M, Wa, and WI-61 cloned into pGS62 (constructed by Prof.

M. McCrae), (iv) pSC11-30R.2 [derived from pSC11 (Chakrabarti *et al.*, 1985) by insertion of an oligonucleotide at the SmaI site containing an initiator AUG downstream of which are three unique restriction enzyme sites (NcoI, SmaI and StuI) providing for insertion of foreign gene fragments in each reading frame and finally stop codons in each of the three frames], and (v) rotavirus UKtc gene 8 fragments (VP7 protein) ClaI-HhaI and HinfI-EcoRV cloned into pSC11-30R.2 (constructed by F. Xu). All cloning was performed in the rotavirus laboratory, University of Warwick.

4.1.5 Bacterial strains

The bacterial strains employed during the cloning of rotavirus genes were *E. coli* strain INVαF` from Invitrogen's TA Cloning™ system and *E. coli* strain MC1061 obtained from Prof. M. McCrae, University of Warwick.

Table 5. Sequence of Oligonucleotide Primers. Sequence in bold shows region of the primer that is complementary to the rotavirus gene 11 sequence.

Primer name	Sequence
5' gene11	5' TCCGGATCCAGATCTGGCCAT**GGCTTTTAAAGCGCT** 3'
3' gene11	3' **GGGCCCGGGCACGTGGCCAGCTGCAGGTCACAAAACGGGA** 5'
5' gene11(orf2)	5' TCCGGATCCAGATCTAGACGTCG**ACGTGACGAGTCTTC** 3'
3' gene11(orf2)	3' **GGGCCCGGGCATGCGGCCGCGGTACCTAGACACGCCAGCG/AT** 5'
5' $P_{7.5}$	5' CATATGCCGGTAGTTGCGAT 3'
5' TK	5' GAACGGCGGACATATTCAGT 3'
3' TK	3' CATCGAGTGCGGCTACTATA 5'

4.1.6 Rotavirus strains

The bovine strain Compton UK tissue adapted (UKtc) (serotype 6) was originally provided by Dr. M. Thouless, East Birmingham Hospital, Birmingham.

The simian strain RRV (serotype 3) was originally provided by Dr. D. Snodgrass, Moredun Research Institute, Edinburgh.

Rotavirus was pre-treated with 10 μg/ml trypsin for 30 min at 37°C, and then propagated in the presence of 2 μg/ml trypsin.

4.1.7 Vaccinia virus

Recombinant vaccinia viruses were termed 'vacc' followed by the rotavirus strain, protein number and restriction enzyme fragment, where appropriate. VaccUKtc.VP1, NS53, NS34, NS35 and NS28 were produced by S. Stagg, and vaccUKtc.VP4, VP6 and VP7 were produced by R. Jenkins. VaccUKtc.VP7(DraI-EcoRV) and vaccUKtc.VP7(EcoRV-HincII) were produced by F. Xu. All these virus recombinants were made in the rotavirus laboratory, University of Warwick.

The wild-type vaccinia virus used was the Western Reserve strain WR^{+} and was provided by Prof. M. McCrae, University of Warwick.

4.1.8 Animals

Balb/c ($H\text{-}2^{d}$) and C3H/He,mg ($H\text{-}2^{k}$) mice were obtained from breeding colonies within the Department of Biological Sciences, University of Warwick. Balb,B ($H\text{-}2^{b}$), C57BL/6 ($H\text{-}2^{b}$), C57BL/10 ($H\text{-}2^{b}$), B10.D2/n ($H\text{-}2^{d}$) and B10.BR ($H\text{-}2^{k}$) mice were all purchased from Harlan UK limited, Bicester, Oxon. All mice were female and 6 to 10 weeks old when sacrificed. Mice were housed in negative pressure isolation units after inoculation with rotavirus.

4.1.9 Suppliers

General chemicals were obtained from **BDH Laboratory Supplies (Merck Ltd., Poole, Dorset), Fisons Scientific Equipment (Loughborough, Leics.)**, and **Sigma-Aldrich Company Ltd. (Poole, Dorset)**, and were of molecular biology or analytical grade.

Equipment and other reagents (unless otherwise stated) were obtained from the following suppliers: **Amersham International plc. (Little Chalfont, Bucks.)**, Biotinylated goat anti rabbit IgG (H+L), Streptavidin fluorescein, ^{51}Chromium-aqueous, **Beckman Instruments Ltd. (High Wycombe, Bucks.)**, centrifuge tubes, **Becton Dickinson Ltd. (Oxford, Oxon.)**, syringes, **Difco Laboratories (Basingstoke, Hants.)**, Bacto-agar, Bacto-tryptone, yeast extract, **Gibco-BRL Life Technologies Ltd. (Paisley, Scotland)**, 1 Kb DNA ladder, restriction and modification enzymes, Lipofectin™, Dutch modified RPMI 1640 medium, Geneticin G418 sulphate, HAT supplement (50X) liquid, **Invitrogen (R&D Systems Ltd., Abingdon, Oxon.)**, TA Cloning™ System, electroporation cuvettes and **Nunclon (High Wycombe, Bucks.)**, 75 cm^2 and 150 cm^2 tissue culture flasks, 6-, 12-, 24-well tissue culture dishes and 96-well plates.

4.2 CELL CULTURE

4.2.1 Cell maintenance

Cultured cells were maintained at 37°C in either a 5% CO_2 incubator in a humidified atmosphere (for flasks) or on a rotary rolling bottle apparatus (Modular Cell Production, Model III) (for roller bottles), using the appropriate media (see section 4.1.2). 150cm^2 flasks and roller bottles of monolayer cells were passaged when confluent using trypsin/Versene (1:5) to detach the cells. 75 cm^2 flask suspension cells (EL4 and P815) were subcultured when cell density reached 5 X 10^6 cells/ml. All cells were reseeded at a

ratio of 1:4 with fresh medium and manipulations were carried out under sterile conditions using standard aseptic techniques.

4.2.2 Freezing and recovery of cell stocks

Long-term cell stocks were prepared by suspending the cells from one 150 cm^2 flask in 1.8 ml of appropriate medium (see section 4.1.2) containing 30% FCS and 8% (v/v) dimethylsulphoxide (DMSO). The freezing vials were slowly cooled to -70°C and then immersed in liquid nitrogen.

Cells were recovered by rapidly thawing the contents of one vial and transferring the cells to a 75 cm^2 flask of pre-warmed medium. The medium was changed the following day.

4.3 CLONING TECHNIQUES

4.3.1 Rotavirus RNA extraction

150 cm^2 flask of confluent BS-C-1 cells were inoculated with rotavirus at a multiplicity of infection (moi) of 0.1. The cells were harvested at maximum cytopathic effect (cpe) following further incubation at 37°C, 5% CO_2. The cells were pelleted by centrifugation and resuspended in 5 ml of lysis buffer containing 100 mM Tris-HCl pH 8.0, 50 mM NaCl, 10 mM EDTA and 0.5% Nonidet P40. Following 10 min incubation on ice, the nuclei and unlysed cells were pelleted and the cytoplasmic supernatant collected. The supernatant was then incubated at 37°C for 3 hr following the addition of 0.5 mg/ml proteinase K and 0.5% SDS. The samples were then phenol extracted (three times), ethanol precipitated and resuspended in 50 µl of water.

4.3.2 Quantification of nucleic acids

DNA and RNA were quantified in solution by spectrophotometry. Absorbance readings were taken at 260 nm (A_{260}) where an absorbance of 1.0 was taken as indicating a concentration of 50 μg/ml for double-stranded DNA, or 40 μg/ml for RNA.

Digested DNA was quantified using agarose gel electrophoresis (see section 4.3.4) by comparing the band intensity of 1 μl of resuspended DNA with 200 ng of pGS62 plasmid DNA.

4.3.3 Reverse transcription polymerase chain reaction (RT-PCR)

The reaction was set up in 50 μl containing RNA (approximately 100 ng), 4 μl of primer mixture (100 ng/μl) (see section 4.1.3) and 4 μl DMSO. This solution was then overlaid with 40 μl of liquid paraffin and subjected to 95°C for 2 min and 42°C for 2 min. 50 μl 2X RT-PCR reaction mixture (see section 4.1.1) was added to the solution and the incubation continued at 42°C for 33 min. 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 3 min then followed using a DNA Thermal Cycler (Perkin Elmer Cetus).

4.3.4 Agarose gel electrophoresis

1.0% agarose in 1X TAE or TBE buffer (see section 4.1.1) was heated in a microwave for a few minutes and then cooled to about 50°C. 12.5 μg ethidium bromide was added to the agarose before pouring.

DNA samples (and the routinely used 1 Kb ladder) were prepared by the addition of 10X loading buffer (see section 4.1.1) before being loaded into the preformed wells. Electrophoresis was performed at 90 mA for 60 min and the gels were photographed under UV light using Polaroid 665 or 667 film.

4.3.5 Purification of DNA from agarose gels

A small quantity of agarose gel containing the required DNA fragment was prepared using a scalpel and the DNA was then purified using a Bio101 Gene Clean® II purification kit (Stratech Scientific, Luton, Beds.). The manufacturers recommendations were followed.

4.3.6 Direct cloning of PCR products

The Invitrogen TA Cloning™ System allowed for direct cloning of PCR products into the pCR™ II cloning vector. The manufacturer's recommendations were followed.

4.3.7 Mini-plasmid preparation

Mini-plasmid preparations (Maniatis *et al.*, 1982) were used in the screening of transformants to prepare DNA for restriction enzyme digestion analysis.

4.3.8 Restriction enzyme digestion

Restriction enzyme digests were carried out under the conditions recommended by the suppliers. All reactions were carried out at 37°C.

4.3.9 Maxi-plasmid preparation

Plasmid DNA from positive transformants was amplified by maxi-preparation using the method described by Maniatis *et al.* (1982).

4.3.10 Blunt ending of DNA

The 5' overhangs on digested DNA were removed using T4 DNA polymerase. A 20 µl volume containing digested DNA, 1X T4 polymerase buffer, 2 µl of 1 mM dNTPs and 1 µl (0.1 units) T4 DNA polymerase was incubated at 11°C for 20 min. The enzyme was inactivated by the addition of 2 µl 0.5M EDTA.

4.3.11 Dephosphorylation of vector DNA

10 μg of restriction endonuclease digested DNA was resuspended in 200 μl of 1X calf intestinal phosphatase (CIP) buffer (0.5M Tris-HCl pH 9.0, 10 mM $MgCl_2$, 1 mM $ZnCl_2$ and 10 mM Spermidine), including 1 μl CIP (1 unit/μl), and incubated at 37°C for 15 min followed by 60°C for 15 min. A further 1 μl of enzyme was added and the incubation repeated as before. The CIP was inactivated by the addition of 2 μl 0.5M EDTA. Samples were then phenol extracted (three times), ethanol precipitated and resuspended in 50 μl of water.

4.3.12 DNA sequence analysis

DNA cycle sequencing was carried out using the Applied Biosystems 373A (version 1.2.0) Automated Sequencer by L. Ward, Department of Biological Sciences, University of Warwick, according to manufacturer's instructions.

4.3.13 Ligation of DNA

DNA fragments were ligated into phosphatased vector. Ligation was allowed to proceed at 15°C overnight and contained DNA (insert (20): vector (1) mix), 10X ligation buffer (0.66M Tris-HCl pH 7.6, 50 mM $MgCl_2$, 50 mM DTT, 100 mM ATP) and 1 unit T4 DNA ligase, in a total volume of 15 μl. The DNA ligation mixture was ethanol precipitated and the vacuum dried pellet resuspended in 10 μl of distilled water.

4.3.14 Electroporation

Ligated DNA samples were electroporated into competent cells of the *E. coli* strain MC1061 following the methods described in Maniatis *et al.* (1982). The cells were pulsed in a BIORAD Gene Pulser at 2.5 kV, with 25 μF set as capacitance and 200Ω on the pulse

controller. Cells were finally plated onto L-agar plates containing 100 μg/ml ampicillin. The plates were incubated at 37°C overnight and the DNA from potential clones was amplified using mini-plasmid preparation (see section 4.3.7).

4.3.15 Polymerase chain reaction (PCR)

A PCR was performed in a reaction volume of 25 μl. This contained DNA (approximately 100 ng), 1 μl of primer mixture (100 ng/μl) (see section 4.1.3) and 1X PCR reaction mixture (see section 4.1.1). The solution was overlaid with 25 μl of liquid paraffin and subjected to 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 3 min. 10 μl of the reaction was analysed by agarose gel electrophoresis (see section 4.3.4).

4.4 CONSTRUCTION AND EXPRESSION OF RECOMBINANT VACCINIA VIRUS

4.4.1 Transfection

Approximately 90% confluent monolayers of CV-1 cells in 6-well tissue culture dishes were washed two times with PBS. Wild-type vaccinia virus (WR^+) was added to the cells at an moi of 0.005 in a volume of 200 μl of PBS and the cells were incubated at 37°C, 5% CO_2 for 90 min with gentle rocking every 20 min.

Approximately 20 min before the end of the infection period, a transfection solution was prepared as follows: 15 μl of 1 mg/ml lipofectin™ was diluted into 1 ml of GMEM (no FCS) in a polystyrene tube. The tube was vortexed before 5 μg of plasmid DNA was added and the tube vortexed again. This solution was kept at room temperature for 15 min before being used.

The virus inoculum was aspirated from the cell monolayer and the cells washed once in GMEM (no FCS). The plasmid DNA/lipofectin™ complex was added to each well of infected cells and the plates were returned to the 37°C, 5% CO_2 incubator for 4 hr. 1 ml

of GMEM (10% FCS) was then added to each well and, after a further 48 hr incubation, the cells and medium were harvested using a rubber policeman and stored at -70°C.

4.4.2 Selection of recombinants

The TK selection method (Mackett *et al.*, 1984) was used to screen for recombinant vaccinia virus. Transfected cells were freeze-thawed three times and sonicated for 30 sec to release virus. 10 and 100-fold dilutions were made in PBS. Confluent monolayers of HuTK$^-$ 143B cells in 6-well tissue culture dishes were washed in PBS (twice) and 200 µl of diluted virus was added to each well. Dishes were incubated at 37°C for 1 hr with gentle rocking every 20 min. Cells were then overlaid with 4 ml of GMEM (5% FCS) containing 1.4% agar and 50 µg/ml 5'-bromodeoxyuridine (BrdU). Cells were incubated for 3 days before 0.1% neutral red (1:3 diluted in PBS) was placed upon the agar overlays. After 4 hr incubation in the dark, the neutral red was removed and the dishes transferred to 4°C for 30 min. Up to 20 isolated virus plaques were picked from each transfection, resuspended in 500 µl of GMEM (no FCS) and stored at -70°C.

4.4.3 Extraction of vaccinia virus DNA

Each well of a 12-well tissue culture dish of sub-confluent monolayer CV-1 cells were inoculated with 1/5 of a virus plaque (100 µl) and incubated at 37°C, 5% CO_2. After 2 days the cells were pelleted by centrifugation, resuspended in 200 µl of lysis buffer and treated as in section 4.3.1.

4.4.4 Immunofluorescence of recombinant vaccinia viruses

Confluent BS-C-1 cells, grown on glass coverslips in 12-well tissue culture dishes, were infected with UKtc rotavirus (at an moi of 0.1), wild-type vaccinia virus (WR^+) or recombinant vaccinia virus (at an moi of 0.002). At 1 hr post-infection, cells were washed

and overlaid with 2 ml of GMEM (5% FCS). After a further 6 hr incubation at 37°C, the cells were fixed for 10 min with ice cold acetone/methanol (1:1) and air-dried before washing twice in PBS.

50 µl of primary antibody diluted 1/100 in PBS, rabbit anti-UKtc rotavirus third bleed (#2148α UKtc, provided by Dr. S. Thomas, University of Warwick), was added to the cells and incubated at 37°C for 1 hr before washing twice in PBS. 50 µl secondary antibody diluted 1/200 in PBS, biotinylated goat anti rabbit IgG (H+L), was added to the cells and incubated at 37°C for 1 hr before washing twice in PBS. 50 µl streptavidin fluorescein diluted 1/400 in PBS was added to the cells and incubated at 37°C for 10 min before washing twice in PBS. The cover-slips were inverted and mounted onto slides in 80% glycerol, 20% PBS and viewed under a UV microscope.

4.5 VIRUS PROPAGATION

4.5.1 Plaque purification of vaccinia virus

Three wells of confluent monolayer CV-1 cells in a 6-well tissue culture dish were infected with 2/5 of a virus plaque (200 µl), 10^{-1} and 10^{-2} dilutions of the plaque, respectively, for 1 hr at 37°C and then overlaid with 4 ml of GMEM (5% FCS) containing 1.4% agar. The cells were incubated for 3 days before 0.1% neutral red (1:3 diluted in PBS) was placed upon the agar overlays. After 4 hr incubation in the dark, the neutral red was removed and the dishes transferred to 4°C for 30 min. Two isolated virus plaques were picked, resuspended in 500 µl of GMEM (no FCS) and three times freeze/thawed at -70°C. This procedure was repeated twice such that all vaccinia virus recombinants had been plaque purified three times before being propagated into virus stocks.

4.5.2 Preparation of vaccinia virus inoculum

Confluent monolayer CV-1 cells in a 6-well tissue culture dish were infected with 2/5 of a plaque purified virus (200 µl) for 1 hr at 37°C and 4 ml of GMEM was then added. The cells were incubated at 37°C until full cpe was observed and the cells were harvested into the growth medium. Confluent CV-1 monolayers in 150 cm^2 flasks were then infected with the harvested virus in a total of 20 ml GMEM (5% FCS). Following complete cpe the virus was harvested.

4.5.3 Large scale growth of vaccinia virus

Virus was propagated using confluent CV-1 monolayers grown in two roller bottles. The cells were infected at an moi of ≤ 0.1 and the virus inoculum was made up to 25 ml/bottle with GMEM (5% FCS). Virus was harvested following complete cpe and centrifuged at 25,000 rpm, 4°C for 90 min in a Beckman SW28 rotor. Pellets were resuspended in 6 ml of GMEM (no FCS) and stored at -70°C until use.

4.5.4 Preparation of rotavirus inoculum

Confluent BS-C-1 monolayers in 150 cm^2 flasks were washed with PBS, infected with trypsin treated rotavirus at an moi ≤ 0.1 and made up to 15 ml with GMEM (no FCS). Virus growth was allowed to proceed for 3 to 5 days until complete cpe was observed. Virus was harvested and stored at -70°C.

4.5.5 Large scale growth of rotavirus

Trypsin treated rotavirus was propagated using confluent BS-C-1 monolayers grown in 30 roller bottles. The cells were infected at an moi of ≤ 0.1 and the virus inoculum was made up to 25 ml/bottle with GMEM (no FCS). Virus was harvested once complete cpe was

observed. Aprotinin (4 μg/ml) was added to the harvested virus to stabilise the double-shelled virus particles (Keljo and Smith, 1988).

4.5.6 Rotavirus purification

The viral and cellular debris were concentrated by centrifugation at 25,000 rpm, 4°C, for 90 min in a Beckman SW28 rotor. Pellets were resuspended in virus resuspension buffer (50 mM Tris-HCl pH 8.0, 10 mM NaCl, 1.5 mM β-mercaptoethanol and 3 mM $CaCl_2$) and then homogenised with 1/10 volume of Arcton 113 (trifluoroethane) three or four times using a Virtis 123 homogeniser (3 min at half speed). The two phases were separated by low speed centrifugation (500 Xg, 4°C for 20 min). The aqueous phases from each extraction were pooled and back extracted with 1/15 volume of Arcton. The virus was concentrated by centrifugation at 25,000 rpm, 4°C for 2 hr in a Beckman SW28 rotor. The pellets were allowed to resuspend overnight at 4°C in 50 mM Tris-HCl pH 8.0.

After resuspension by sonication, virus was loaded onto preformed ρ=1.2 to 1.4 caesium chloride density equilibrium gradients. These were centrifuged at 20,000 rpm, 4°C for 2 hr in a Beckman SW28 rotor. Double-shelled particles (ρ=1.36) and single-shelled particles (ρ=1.38) were collected together by side puncture, diluted in 50 mM Tris-HCl pH 8.0, and re-centrifuged as described for concentrating virus. The virus pellet was resuspended in 500 μl of Tris-HCl pH 8.0 and stored at -70°C until use.

4.5.7 Titration of virus stocks

CV-1 cells were used for vaccinia virus, whereas, BS-C-1 cells were used for trypsin treated rotavirus. Serial 10-fold dilutions were prepared in PBS and 200 μl inoculated onto cells in 6-well tissue culture dishes. After incubation at 37°C for 1 hr, the cells were overlaid with GMEM (5% FCS) containing 1.4% agar and incubated for a further 4 days. The cells were fixed with formal-saline (30% formaldehyde in PBS v/v) for 5 hr and the

agar overlayers removed. The monolayers were then stained with 7.5% crystal violet for a few minutes and excess stain was removed with water. Clearly visible plaques were counted and the titre calculated.

4.6 CHROMIUM-51 (^{51}Cr)-RELEASE ASSAY TO MEASURE CTL ACTIVITY

4.6.1 *In vivo* priming of mice

Two mice (see section 4.1.8) were inoculated orally with purified, trypsin treated rotavirus of known titre [routinely 10^7 plaque forming units (pfu)] by proximal oesophageal intubation through a 2 inch 18 gauge metal gavage (IMS, Cheshire).

4.6.2 Preparation of spleen cell suspension

Mice were sacrificed 7 days after oral inoculation and the spleens removed. Single-cell suspensions were obtained by teasing the splenocytes through a 200 mesh stainless steel gauze using a 5 ml plastic syringe plunger. The splenocytes were washed twice in RPMI DM (5% FCS) by centrifuging at 900 rpm for 5 min in a bench centrifuge (IEC Centra-7R) at room temperature. Red blood cells were removed using 2 ml of ice-cold water for 4 sec and the splenocytes were washed twice before being resuspended in 10 ml of RPMI DM (10% FCS) (responders).

4.6.3 *In vitro* stimulation of splenocytes

Splenocytes from primed mice (responders) were stimulated *in vitro* with rotavirus-infected, syngeneic splenocytes from unprimed mice (stimulators). The priming virus, at an moi of 1, was added to 2 X 10^7 stimulators in a volume of 1 ml RPMI DM (no FCS) for 1 hr at 37°C, 5% CO_2. Using RPMI DM (10% FCS), responders were adjusted to 6 X 10^6 cells/ml and stimulators were adjusted to 3 X 10^6 cells/ml. 1ml of both responders and stimulators were added to each well of a 24-well tissue culture plate. The final

concentration of cells per well was 9 X 10^6 and the ratio of responders to stimulators was 2:1. The cells were cultured for 6 or 7 days in a 37°C, 5% CO_2 incubator (effectors).

4.6.4 Preparation of target cells

Target cells (EL4, Ltk^-, L-D^b, L-K^b or P815) were washed once by centrifugation [1500 rpm for 5 min in a bench centrifuge (IEC Centra-7R) at room temperature] and resuspended in 10 ml of RPMI DM (10% FCS) before being counted. 1 X 10^6 cells were pelleted (as before) and the supernatant discarded. Wild-type vaccinia virus (WR^+) or recombinant vaccinia virus was added to the resuspended cells at an moi of 10 along with 50 µCi of ^{51}Cr in a total volume of 500 µl RPMI DM (10% FCS). The targets were incubated at 37°C, 5% CO_2 for 90 min with occasional shaking of the tube by hand. Following this, excess ^{51}Cr and virus were removed by washing three times (as before) in RPMI DM (5% FCS). The targets were resuspended in 3 ml of RPMI DM (10% FCS) and incubated at 37°C, 5% CO_2 for a further 3.5 hr. The targets were again washed three times and resuspended in 10 ml RPMI DM (10% FCS) to give a final concentration of 1 X 10^5 cells/ml.

4.6.5 ^{51}Cr-release assays

In vitro primed splenocytes (effectors) were harvested by gentle pipetting, centrifuged at 900 rpm for 5 min [bench centrifuge (IEC Centra-7R) at room temperature] and resuspended in 3 ml of RPMI DM (10% FCS). The effectors were counted and adjusted to 3 X 10^6 cells/ml with RPMI DM (10% FCS) to achieve a starting ratio of 30:1 effector cells per target cell. 100 µl of effectors were then added to an equal volume of targets (invariably 1X10^4 cells) in a 96-well round bottomed plate at ratios of 30:1, 10:1, 3:1 and 1:1 in triplicate (for test ^{51}Cr release). Twelve wells of the 96-well plate were left free of effector cells. To six of these wells, 100 µl of 5% Triton X-100 was added (for total ^{51}Cr

release) and to the other six wells, 100 µl of RPMI DM (10% FCS) was added (for spontaneous ^{51}Cr release). The plates were centrifuged at 900 rpm for 5 min and placed for 5 hr at 37°C, 5% CO_2 in a humidified incubator.

100 µl of supernatant fluids were removed from each well, transferred into LP-2 tubes (Luckhams) and sealed with 100 µl of molten paraffin wax. Radioactivity was measured for 1 min using a γ-radiation counter (Packard Cobra auto-gamma).

4.6.6 Calculation of the percentage specific lysis

The percentage of specific lysis at each effector:target ratio was calculated using the following formula:

$$\% \text{ specific lysis} = 100 \text{ X} \quad \frac{\text{test } ^{51}Cr \text{ released} - \text{spontaneous } ^{51}Cr \text{ released}}{\text{total } ^{51}Cr \text{ released} - \text{spontaneous } ^{51}Cr \text{ released}}$$

Positive CTL killing of target cells (infected with recombinant vaccinia virus) was defined by (i) a minimum 10% specific lysis, and (ii) a minimum threefold greater % specific lysis than the negative control (target cells infected with wild-type vaccinia virus). All assays were performed in triplicate and results were calculated as an average of three values. All experiments were performed at least twice with similar results.

CHAPTER 5

CLONING OF ROTAVIRUS GENE 11

INTO THE pGS62 VACCINIA SHUTTLE VECTOR

5.1 AIMS

The aim of the work described in this chapter was to clone both the full length open reading frame of rotavirus UKtc gene 11 and the second, shorter, open reading frame [gene 11(orf2)] into the vaccinia shuttle plasmid pGS62. This would complete the cloning of the full complement of rotavirus UKtc genes into this vector.

5.2 PCR AMPLIFICATION OF ROTAVIRUS UKTC GENE 11

Primers were designed based on the UKtc gene 11 sequence obtained from the EMBL database. Two sets of primers termed 5' and 3' gene 11 and 5' and 3' gene 11(orf2) (see Table 5) allowed amplification of the whole gene 11 and the second open reading frame (orf2) of gene 11, respectively. Primers were designed with various restriction enzyme sites added to their 5' ends to allow easier handling of the cloned gene 11 DNA.

Two separate RT-PCR reactions were carried out, each using 100 ng of extracted rotavirus UKtc RNA and one of the primer pairs. The amplified DNA obtained was analysed using agarose gel electrophoresis (see Figure 8) and PCR products of the expected size, namely 713 base pairs (bp) for gene 11 and 304 bp for gene 11(orf2) were obtained in these RT-PCR reactions. The amplified cDNA's were excised from the gel and the DNA purified using the Gene Clean® II kit.

5.3 TA CLONING™ OF ROTAVIRUS GENE 11 DNA

The gene 11 PCR products were first cloned using the TA cloning™ system because this kit allows the direct ligation of amplified PCR products into the pCR™ II vector (see Figure 9). Insertional inactivation of pCR™ II leads to a frame shift and the formation of positive white colonies, whereas, negative blue colonies result from a T:T mismatch and self-ligation of the vector.

FIGURE 8. PCR AMPLIFIED ROTAVIRUS UKTC GENE 11 PRODUCTS

Rotavirus UKtc gene 11 and gene 11(orf2) DNA were both amplified by RT-PCR using the protocol described in section 4.3.3 of Materials and Methods. The 5' and 3' gene 11 and gene 11(orf2) primers were used (Table 5) to amplify products of 713 bp and 304 bp, respectively. The products were analysed using a 1% agarose gel. Lane M shows the DNA size markers, lane 1 shows PCR amplified gene 11 and lane 2 shows PCR amplified gene 11(orf2).

FIGURE 10. POTENTIAL GENE 11 CLONES DIGESTED FROM pCR™ II VECTOR USING ECORI RESTRICTION ENZYME

The PCR amplified gene 11 and gene 11(orf2) were directly cloned into pCR™ II plasmid from the TA cloning™ system as described in 4.3.6 of Materials and Methods. Following mini-plasmid preparation, potential clones were screened using restriction enzyme digestion as described in section 4.3.8 of Materials and Methods and the digestion products were analysed using a 1% agarose gel. Digestion with EcoRI was used since the insertion site of the pCR™ II vector is flanked by sequences digested by this enzyme (5 bp either side of the insertion site). EcoRI sites are not present in the inserted sequences or elsewhere in the vector and, consequently, EcoRI digestion resulted in products of 731 bp and 322 bp (including the 5' overhangs produced) for gene 11 and gene 11(orf2) positive clones, respectively. Lane M shows the DNA size markers, lanes 1, 2 and 3 show screening of potential pCR™ II clones containing gene11 (all lanes were deemed positive) and lanes 4 and 5 show screening of potential pCR™ II clones containing gene 11(orf2) (lane 5 was deemed positive).

FIGURE 8

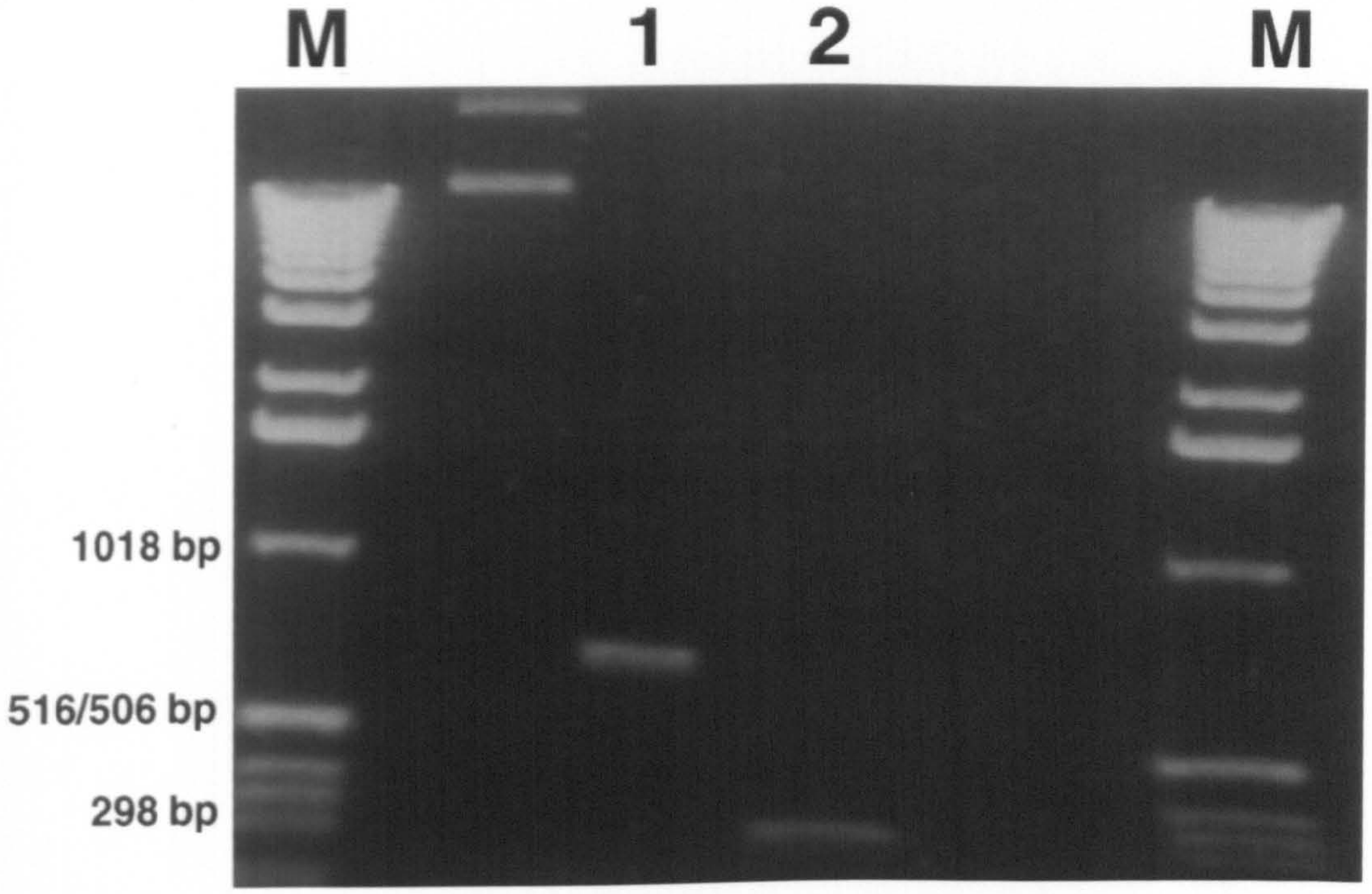


FIGURE 10

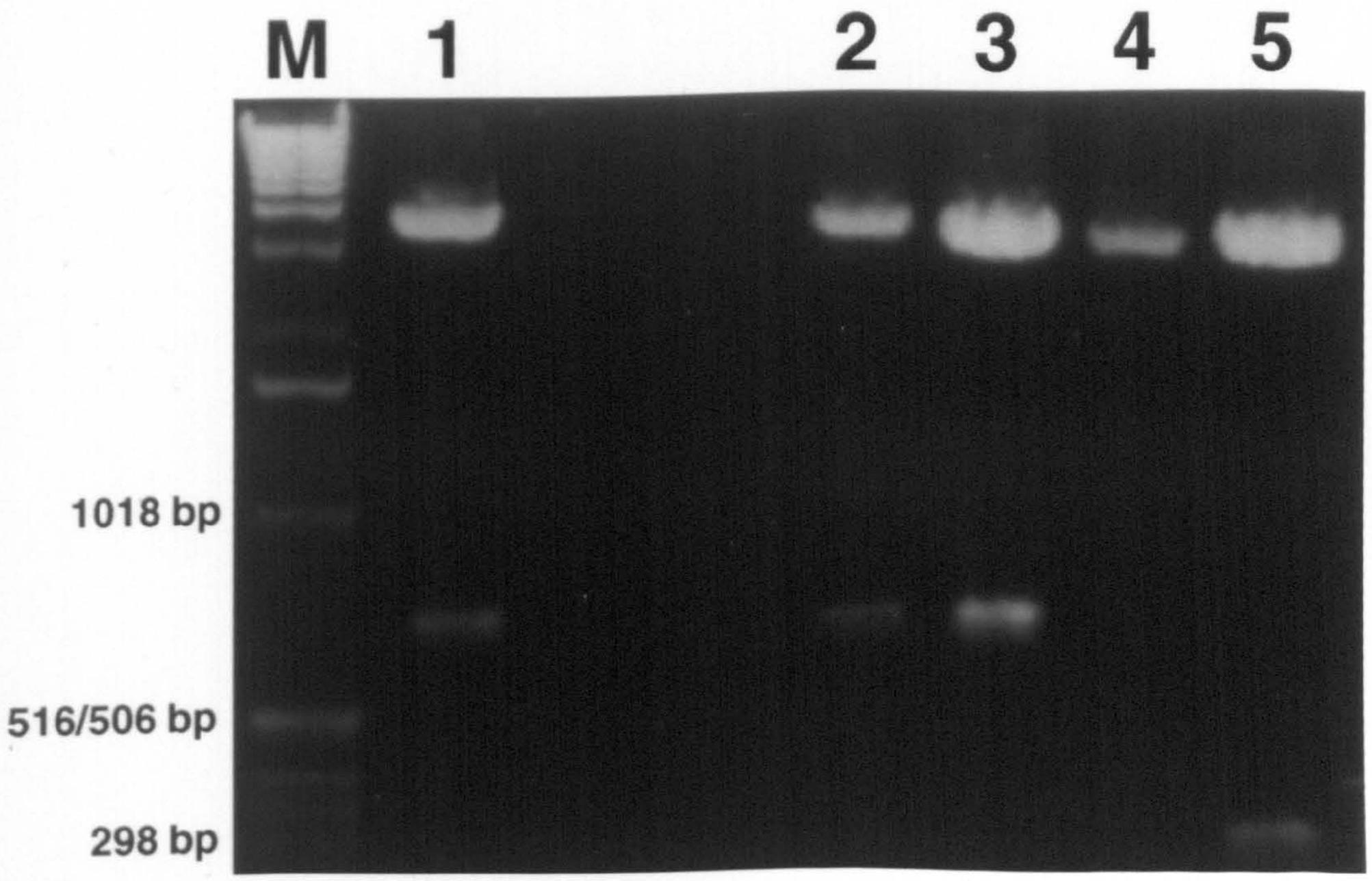
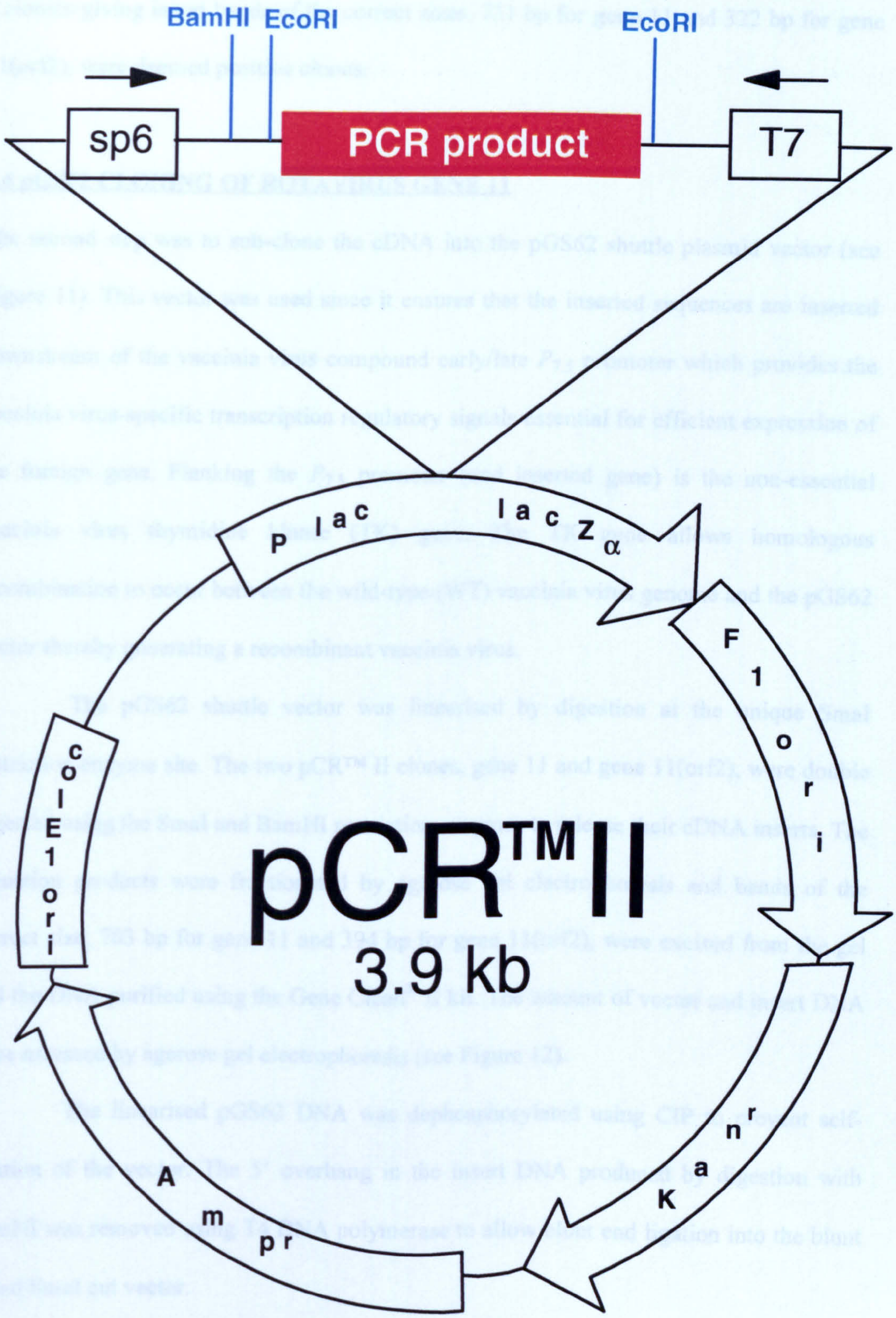


FIGURE 9. MAP OF THE pCR™ II VECTOR FROM TIIE TA CLONING™ SYSTEM

The PCR amplified products (in red) were directly inserted into the pCR™ II vector using the TA cloning™ system. The non-template dependent activity of Taq polymerase used in PCR adds single deoxyadenosines to the 3'-end of all duplex molecules. The pCR™ II insertion site contains 3' T-overhangs which allow direct ligation of the PCR product. Surrounding the PCR product are several restriction enzyme sites (in blue) which allow easier handling of the cloned gene. (Although not employed in this study, the presence of the Sp6 and T7 promoter sequences allow transcription of insert DNA when used in the correct system).

The pCR™ II vector contains a bacterial plasmid component including the *E. coli* DNA origin of replication (ColE1 ori) to allow propagation in *E. coli* and both ampicillin (Amp^r) and kanamycin (Kan^r) resistance genes to allow selection of bacteria carrying the plasmid. The presence of the α-peptide of β-galactosidase and its promoter, lacZα and *P*lac respectively, allow blue/white selection since insertional inactivation leads to a frame shift and the formation of white colonies. Blue colonies result from a T:T mismatch self-ligation of the vector. (Although not used in this study, the F1 origin of replication (F1 ori) allows replication of cloned single strand vector DNA following superinfection of *E. coli* with the F1 helper phage).

FIGURE 9


BamHI
EcoRI
EcoRI
sp6
PCR product
T7
P lac
lac Z α
F 1 ori
col E1 ori
pCR™II
3.9 kb
Amp r
Kan r

The DNA from mini-plasmid preparations of the positive white colonies were digested with EcoRI and analysed using agarose gel electrophoresis (see Figure 10). Colonies giving insert bands of the correct sizes, 731 bp for gene 11 and 322 bp for gene 11(orf2), were deemed positive clones.

5.4 pGS62 CLONING OF ROTAVIRUS GENE 11

The second step was to sub-clone the cDNA into the pGS62 shuttle plasmid vector (see Figure 11). This vector was used since it ensures that the inserted sequences are inserted downstream of the vaccinia virus compound early/late $P_{7.5}$ promoter which provides the vaccinia virus-specific transcription regulatory signals essential for efficient expression of the foreign gene. Flanking the $P_{7.5}$ promoter (and inserted gene) is the non-essential vaccinia virus thymidine kinase (TK) gene. The TK gene allows homologous recombination to occur between the wild-type (WT) vaccinia virus genome and the pGS62 vector thereby generating a recombinant vaccinia virus.

The pGS62 shuttle vector was linearised by digestion at the unique SmaI restriction enzyme site. The two pCR™ II clones, gene 11 and gene 11(orf2), were double digested using the SmaI and BamHI restriction enzymes to release their cDNA inserts. The digestion products were fractionated by agarose gel electrophoresis and bands of the correct size, 703 bp for gene 11 and 394 bp for gene 11(orf2), were excised from the gel and the DNA purified using the Gene Clean® II kit. The amount of vector and insert DNA were assessed by agarose gel electrophoresis (see Figure 12).

The linearised pGS62 DNA was dephosphorylated using CIP to prevent self-ligation of the vector. The 5' overhang in the insert DNA produced by digestion with BamHI was removed using T4 DNA polymerase to allow blunt end ligation into the blunt ended SmaI cut vector.

FIGURE 11. MAP OF THE SHUTTLE PLASMID VECTOR pGS62

The pGS62 vaccinia shuttle vector contains a number of elements. The bacterial component (in blue) was generated from pBR328 and a consists of an origin of replication to allow plasmid propagation in *E. coli* and an ampicillin resistance gene (Amp^r) to allow selection of the bacteria carrying the plasmid. The vaccinia virus component (in black) consists of the compound early/late gene promoter sequence derived from a gene encoding a 7.5-kDa structural protein ($P_{7.5}$) joined to a multi-restriction enzyme linker sequence (in red). These elements have been placed within the vaccinia virus thymidine kinase gene. The $P_{7.5}$ promoter drives the trancription of the foreign gene (normally inserted within the multi-restriction enzyme linker sequence) and the flanking TK components allow recombination, within infected cells, between the shuttle plasmid and the wild-type (WT) vaccinia virus genome. This generates a recombinant vaccinia virus (with a TK^- phenotype) which contains the gene of interest and control elements for its expression during virus replication.

FIGURE 11

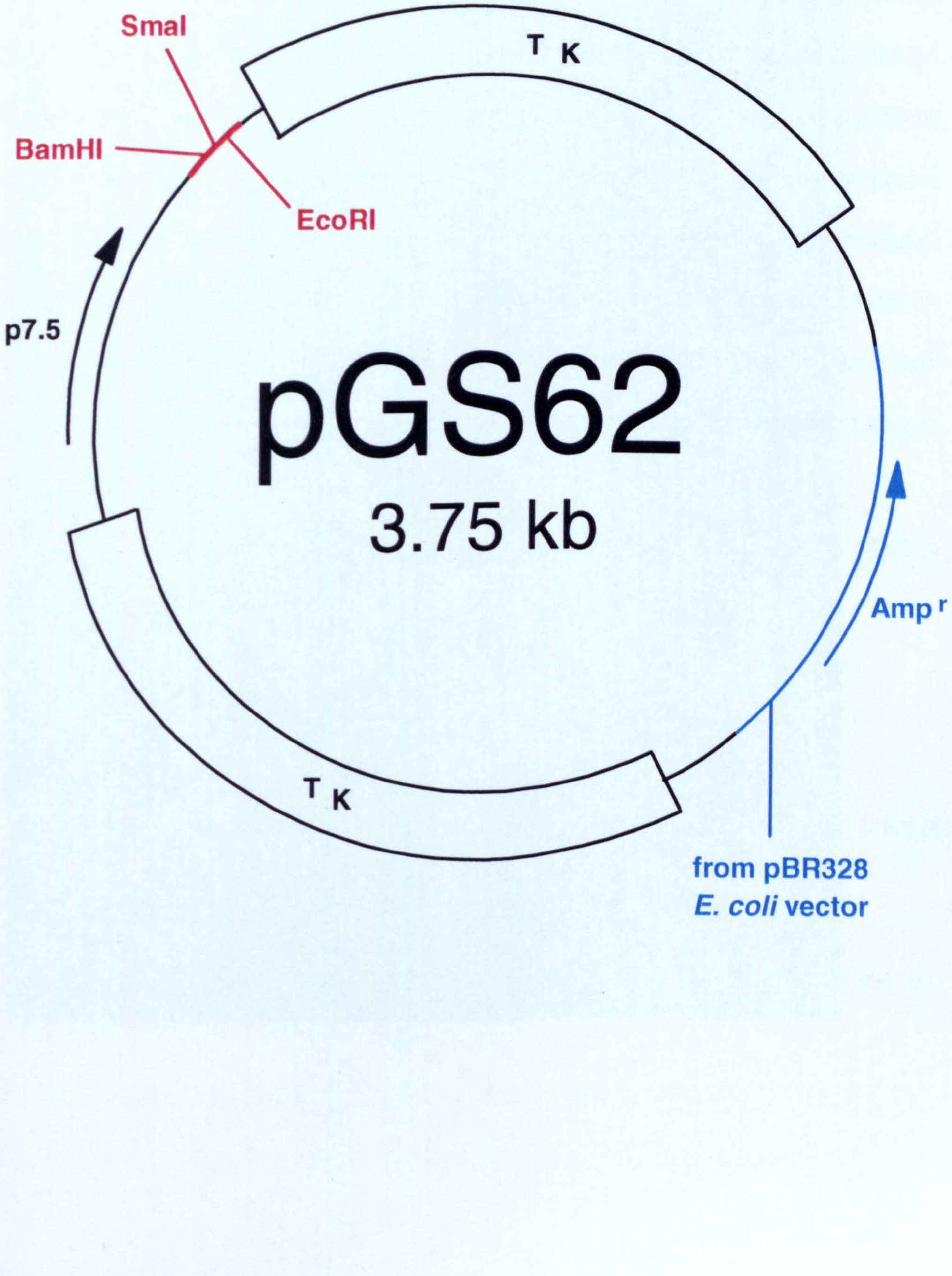
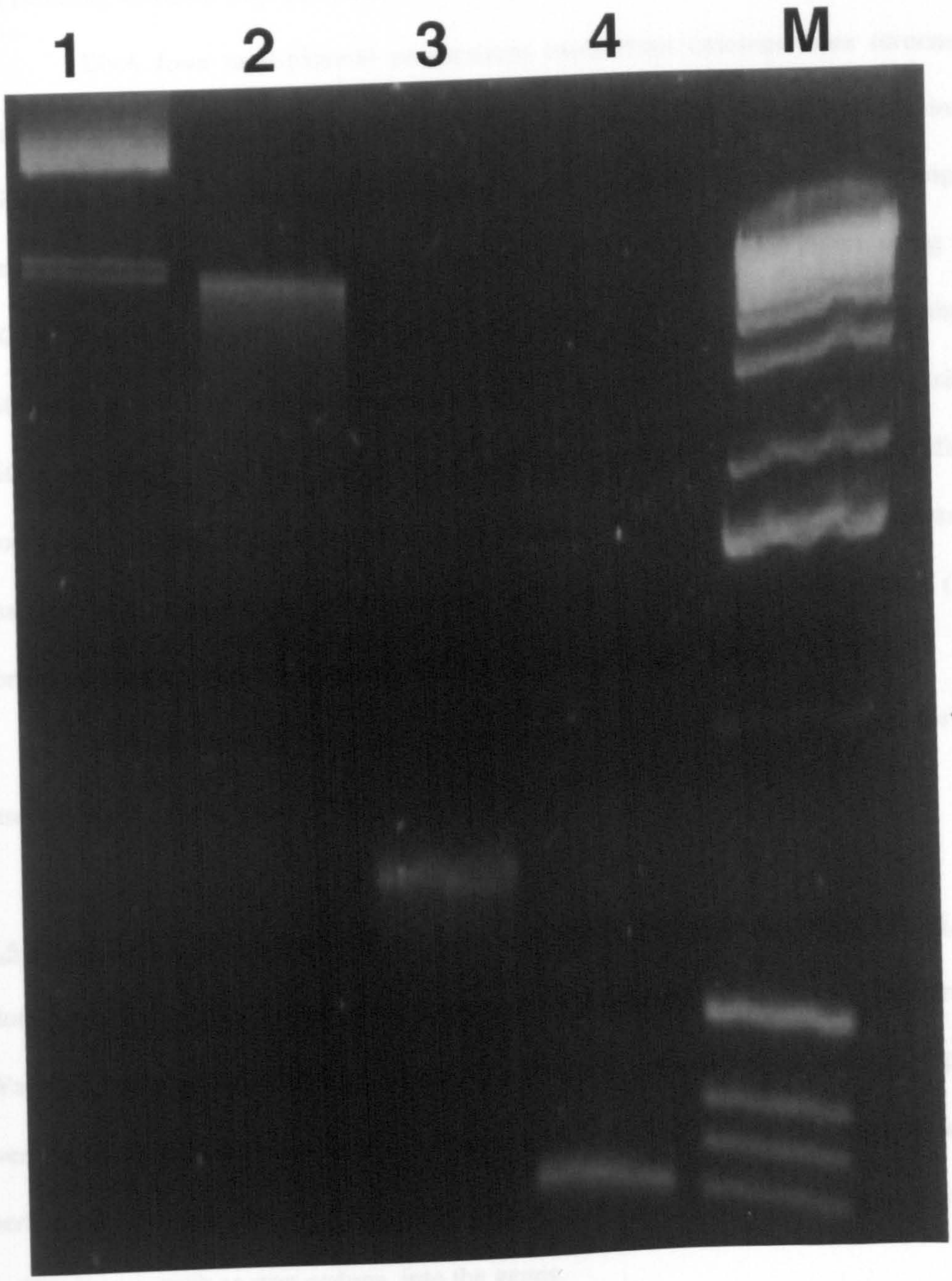

SmaI
BamHI
EcoRI
T K
p7.5
pGS62
3.75 kb
Amp r
T K
from pBR328
E. coli vector

FIGURE 12. QUANTIFICATION OF PCR™ II CLONED GENE 11 INSERTS DIGESTED WITH BAMHI AND SMAI, AND pGS62 VECTOR DIGESTED WITH SMAI

The pGS62 shuttle vector was linearised by digestion at the unique SmaI site as described in Materials and Methods. Double digestion with BamHI and SmaI restriction enzymes of positive pCR™ II clones resulted in products of 703 bp and 394 bp for gene 11 and gene 11(orf2), respectively. The vector and insert fragments were excised from the gel and purified as described in section 4.3.5 of Materials and Methods. The vector and insert DNA was then quantified, following electrophoresis on a 1% agarose gel, by comparing the band intensity of 1 µl of purified DNA with 200 ng of uncut pGS62 (as described in section 4.3.2 of Materials and Methods). Lane M shows the DNA size markers, lane 1 shows 200 ng of uncut pGS62, lane 2 shows 1 µl of linearised pGS62 (~100 ng/µl), lane 3 shows 1 µl of digested gene11 (~100 ng/µl) and lane 4 shows 1 µl of digested gene 11(orf2) (~100 ng/µl).

FIGURE 12.



Digested pGS62 vector was ligated with gene 11 or gene 11(orf 2) insert at a ratio of 20:1. The ligated DNA was then electroporated into competent *E. coli* MC1061 cells. Selection was allowed by the presence of the ampicillin gene in pGS62 and some of the resulting colonies were picked for screening.

DNA from mini-plasmid preparations made from colonies were screened for positive clones by PCR. PCR not only confirmed ligation of the gene 11 inserts into the pGS62 vector but also checked that the insert was in the correct orientation with respect to the vaccinia $P_{7.5}$ promoter within pGS62 (see Figure 13). PCR amplification using the 3' TK and 5' gene 11 or gene 11(orf2) primers (see Table 5) lead to positive PCR products of 760 bp for gene 11 and 351 bp for gene11(orf2). However, due to 5' primer being situated within the insert and the 3' primer being situated within the vector, PCR amplification would only proceed if the insert was in the correct orientation. The PCR products were analysed using agarose gel electrophoresis (see Figure 14) and positive clones (in the correct orientation) were obtained for both gene 11 and gene 11(orf2).

Purified plasmid DNA was prepared from one positive pGS62 clone for either insert using the maxi-plasmid preparation method.

5.5 SEQUENCE ANALYSIS OF pGS62 CLONED ROTAVIRUS GENE 11 DNA

Both gene 11 pGS62 clones were sequenced in duplicate by L. Ward, University of Warwick, using an automated sequencer. The 5' and 3' gene 11 and gene 11(orf2) primers were used to sequence the gene 11 inserts within the pGS62 vector. Sequencing was performed to check that the PCR amplification or cloning steps had not introduced any serious errors, such as stop codons, into the genes.

A single nucleotide change from the published gene 11 sequence was obtained at position 91 (A:T to C:G). This results in an amino acid change at position 30 in gene 11

FIGURE 13. CONFIRMATION OF pGS62 CLONED GENE 11 INSERT DIRECTION BY PCR AMPLIFICATION

PCR was used to confirm that the inserted rotavirus gene 11/gene 11(orf2) fragment (shown in red) had been cloned into the vaccinia pGS62 shuttle vector and that the orientation of insertion was correct with respect to the $P_{7.5}$ vaccinia promoter. This was achieved by using the 5' gene 11 or 5' gene 11(orf2) primer with the 3' TK primer (in blue). Gene 11 insertion in the correct orientation would allow the PCR amplification to proceed giving a product of 760 bp for gene 11 and 351 bp for gene 11(orf2). This includes an additional 50 bp added to the insert size because the 3' TK primer is 50 bp in distance from the insertion position. If there was no insertion, or the inserts were in the wrong orientation, no PCR products would be obtained.

FIGURE 13

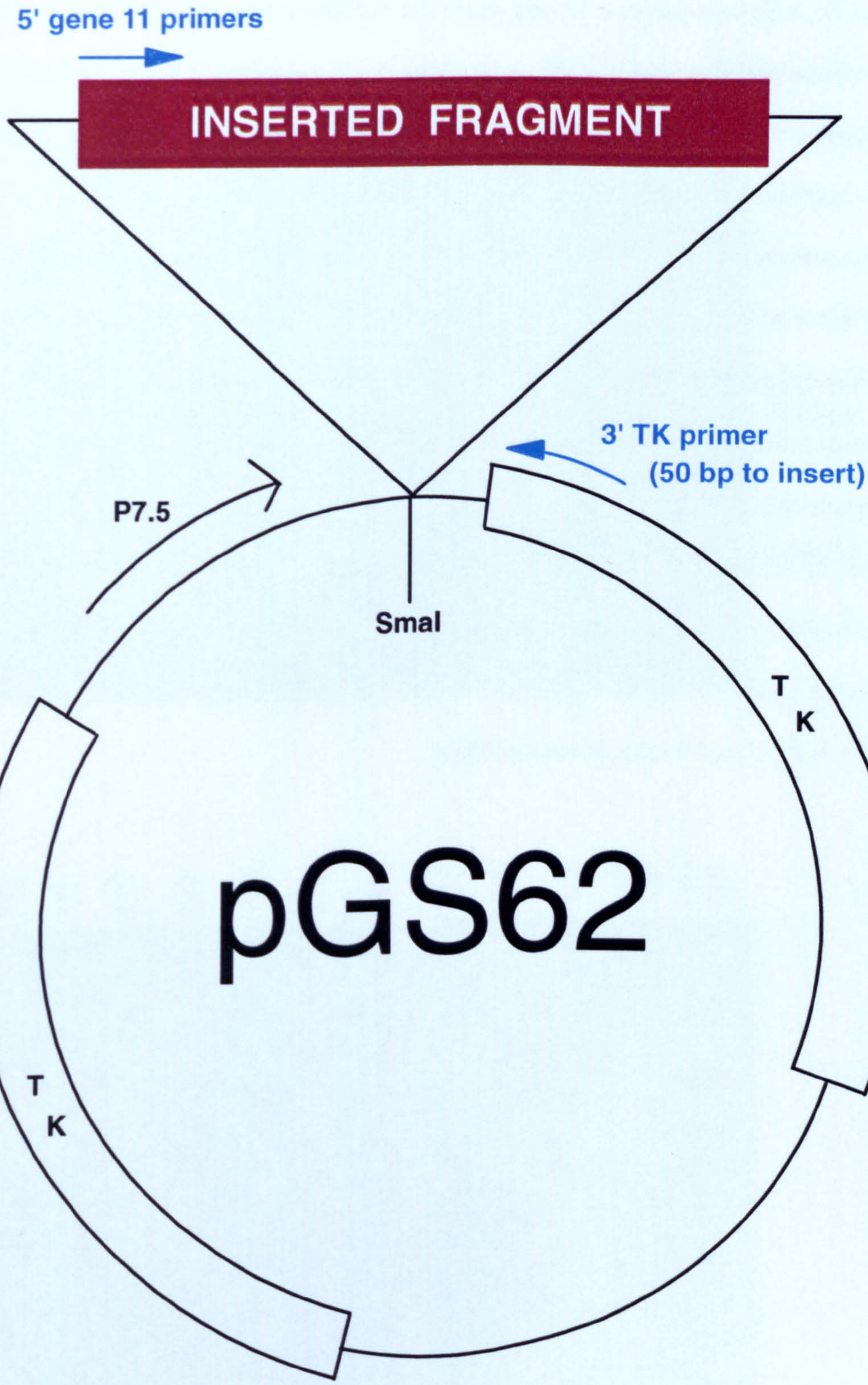

5' gene 11 primers
INSERTED FRAGMENT
3' TK primer
(50 bp to insert)
P7.5
SmaI
T
K
pGS62
T
K

FIGURE 14. PCR AMPLIFIED PRODUCTS OF POTENTIAL pGS62 CLONES CONTAINING ROTAVIRUS UKTC GENE 11 AND GENE 11(ORF2)

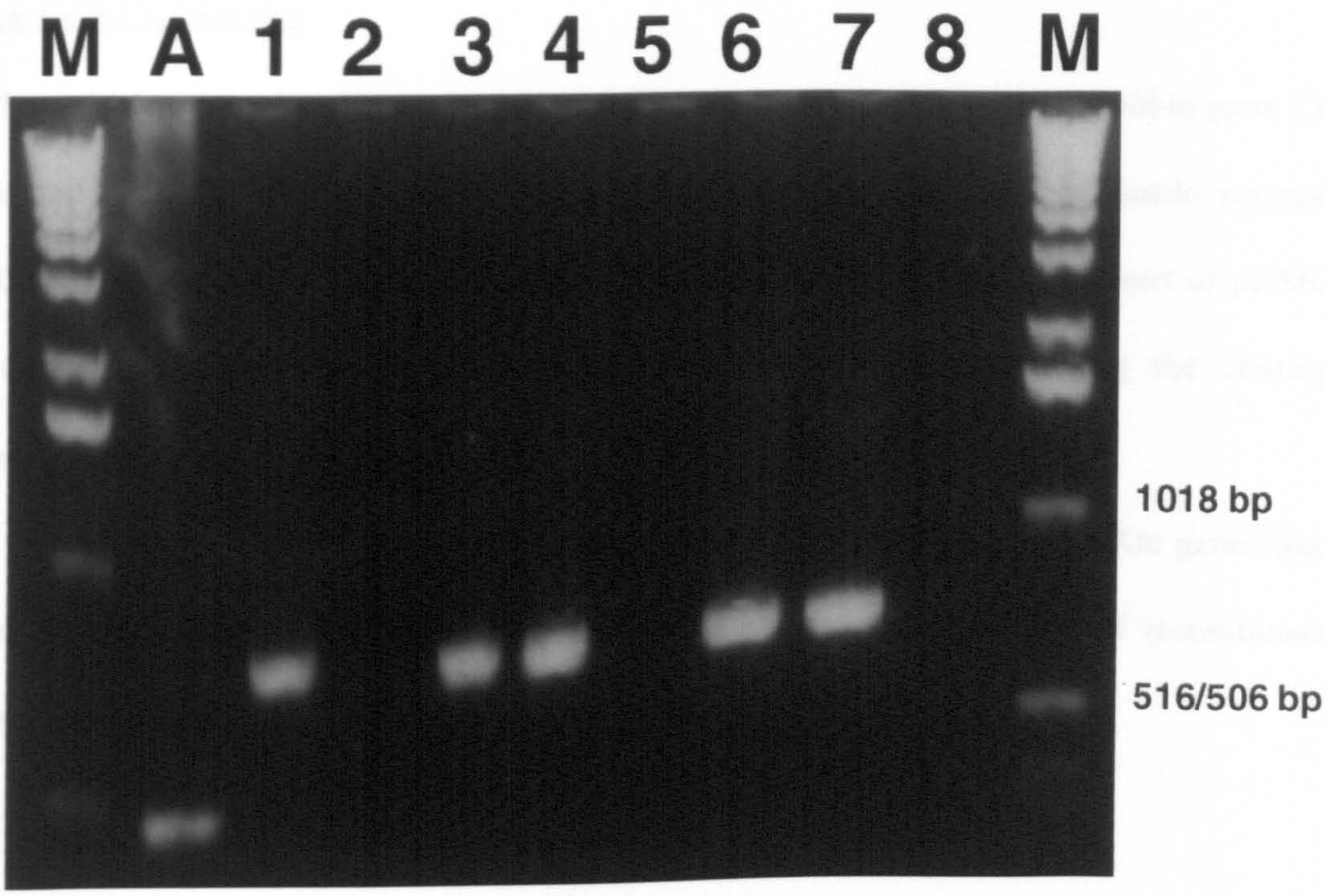
Digested and prepared gene 11 and gene 11(orf2) DNA from the pCR™ II vector were cloned into pGS62 shuttle plasmid at the SmaI site as described in Materials and Methods. Following mini-plasmid preparations, potential clones were screened using PCR amplification as described in section 4.3.15 of Materials and Methods. The 5' gene 11 and 3' TK primers and the 5' gene 11(orf2) and 3' TK primers (Table 5) resulted in products of 760 bp and 351 bp, respectively. The amplified products were analysed using a 1% agarose gel. In both panels lane M shows the DNA size markers and lane A shows the PCR positive control consisting of Adenovirus DNA and a primer pair that gave a product of approximately 500 bp from the left hand end of the Adenovirus genome.

Panel A: Lanes 1 to 8 show screening of potential pGS62 clones containing gene 11 (lanes 1, 3, 4, 6 and 7 were deemed positive).

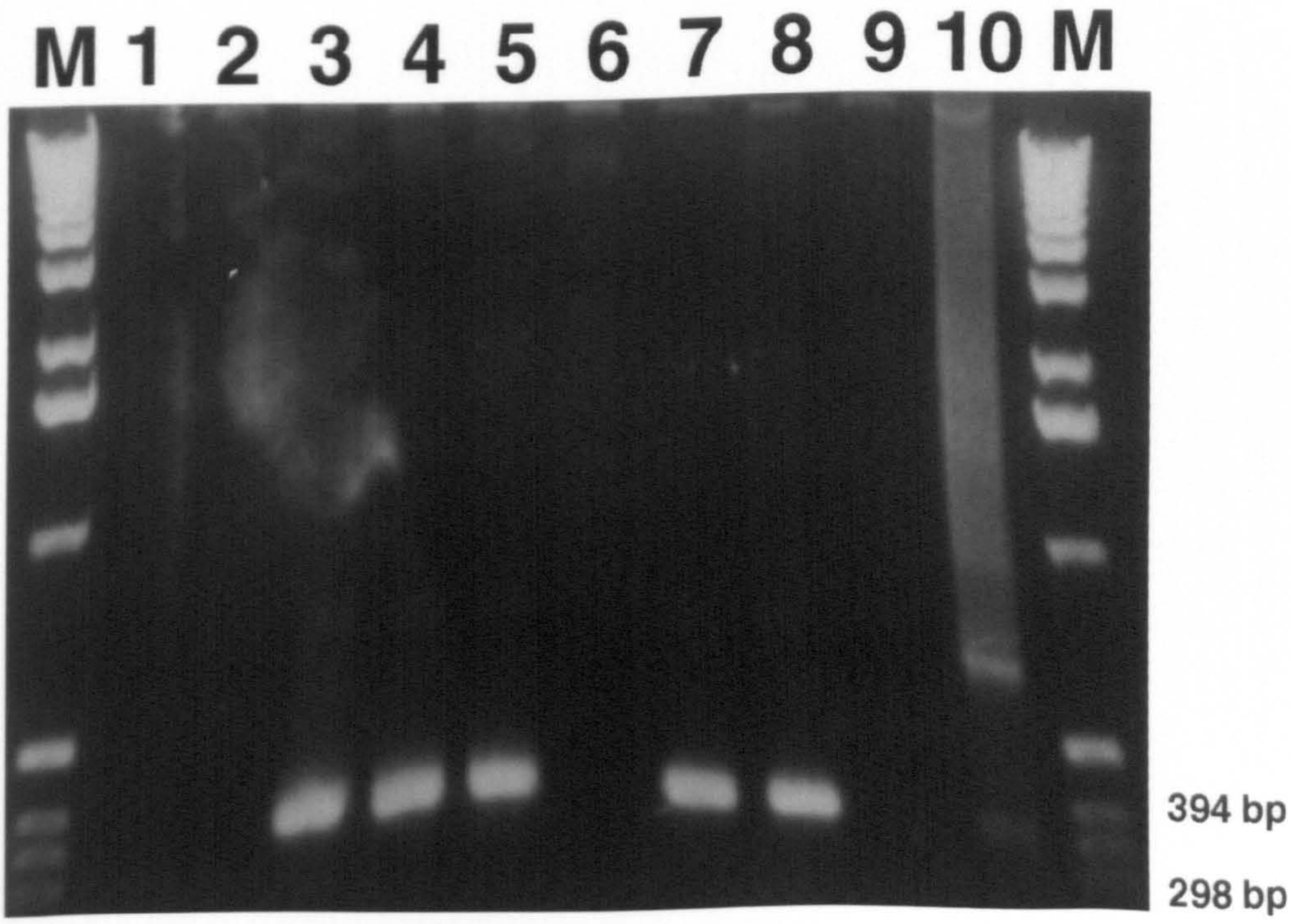
Panel B: Lanes 1 to 10 show screening of potential pGS62 clones containing gene 11(orf2) (lanes 3, 4, 5, 7 and 8 were deemed positive).

FIGURE 14

(A)



(B)



(ser→ pro) (data not shown). No changes were observed in gene 11(orf2) (data not shown).

5.6 CONCLUSIONS

The complete rotavirus UKtc gene 11 and the second open reading frame within gene 11, termed gene 11(orf2), have been successfully cloned into the vaccinia shuttle plasmid pGS62. Sequencing revealed one amino acid change within the gene 11 insert of pGS62 from the published sequence, which may have been introduced during the cloning procedure (aa 30 of gene 11, ser→ pro).

This completed the cloning of the full complement of rotavirus UKtc genes into the pGS62 shuttle vector. This vector, in turn, allowed the construction of recombinant vaccinia viruses.

CHAPTER 6

CONSTRUCTION OF RECOMBINANT VACCINIA VIRUSES

6.1. AIMS

The aim of the work described in this chapter was to use vaccinia shuttle plasmids containing various rotavirus genes (see Table 6) to construct recombinant vaccinia virus containing those genes. The vaccinia virus expression system was used because these viruses have a wide host range. In addition, vaccinia virus DNA replication begins within a few hours post-infection and, thus, foreign proteins are expressed in the infected cell prior to cpe. The infected cells may, therefore, present foreign protein epitopes in association with MHC class I molecules and act as targets during the ^{51}Cr-release assay to measure CTL activity.

Table 6. Constructed vaccinia shuttle plasmids provided

Rotavirus strain	Gene (protein)	Fragment	Plasmid	Constructed by
UKtc	Gene 2 (VP2)		pGS62	Prof. McCrae
UKtc	Gene 3 (VP3)		pGS62	Prof. McCrae
RRV Hochi P9DΔ5	Gene 5 (NS53)		pGS62	Prof. McCrae
RRV A64 Hochi 69M Wa WI-61	Gene 7, 8 or 9 (VP7)		pGS62	Prof. McCrae
UKtc	Gene 8 (VP7)	ClaI-HhaI HinfI-EcoRV	pSC11-30R.2	F. Xu
UKtc	Gene 11 (NS26)		pGS62	R. Heath
UKtc	Gene 11(orf2) (NS12)		pGS62	R. Heath

6.2 PRODUCTION OF RECOMBINANT VACCINIA VIRUSES CONTAINING ROTAVIRUS GENES (USING THE pGS62 VECTOR)

CV-1 cells were infected with wild-type vaccinia virus (WR^+) and transfected with the pGS62 shuttle vector containing an individual rotavirus gene using lipofectin™ (see Table 6). This allowed for homologous recombination to occur between the vaccinia TK gene sequences in the plasmid and virus DNA TK gene sequences (see Figure 15). Following each rotavirus gene transfection, the cells and medium were harvested, freeze-thawed three times and diluted 10- and 100-fold.

The diluted virus samples were plaqued on $HuTK^-$ 143B cells in the presence of 5-bromodeoxyuridine (BrdU). This allowed vaccinia viruses with a TK^- phenotype (and potentially containing the rotavirus gene of interest) to be distinguished from those with a TK^+ phenotype because BrdU is phosphorylated by TK activity and incorporated into the virus genome where it causes lethal mutations. Viruses from up to twenty plaques for each transfected rotavirus gene, deemed TK^- following selection with BrdU, were recovered and resuspended in medium. A fifth of each sample was propagated on CV-1 cells and the DNA extracted when complete cpe was evident.

The extracted DNA was subjected to PCR amplification using the 5' and 3' TK primers (see Table 5). In the case of WT vaccinia virus DNA (which presumably escaped BrdU selection due to mutation in their TK gene), these primers amplified a product including part of the TK gene sequence (300 bp). However, with DNA from positive recombinant vaccinia viruses, these primers amplified a product which included the flanking TK gene sequences (300 bp), the $P_{7.5}$ promoter (322 bp) and the rotavirus gene insert (See Figure 16A and Table 7).

FIGURE 15. GENERATION OF RECOMBINANT VACCINIA VIRUS

Following infection of TK^- cells with WT vaccinia virus, shuttle vector (pGS62 or pSC11-30.R2) containing a foreign gene was transfected into the cell. A double cross-over event occurs between the homologous TK sequences of the vaccinia virus genome and the shuttle plasmid. This homologous recombination results in the insertion of the foreign gene and its control elements into the vaccinia virus genome rendering it TK^-. Recombinant vaccinia viruses with a TK^- phenotype are then distinguished from WT vaccinia viruses with a TK^+ phenotype using the BrdU screening procedure. A TK^- cell line is infected with potential recombinant vaccinia virus diluted 10 and 100-fold and overlaid with agar containing BrdU. The WT vaccinia virus active TK gene phosphorylates BrdU which is then incorporated into the WT vaccinia virus genome where it causes lethal mutations. Thus, following incubation, the visible virus plaques have a TK^- phenotype and probably contain the foreign gene of interest.

FIGURE 15

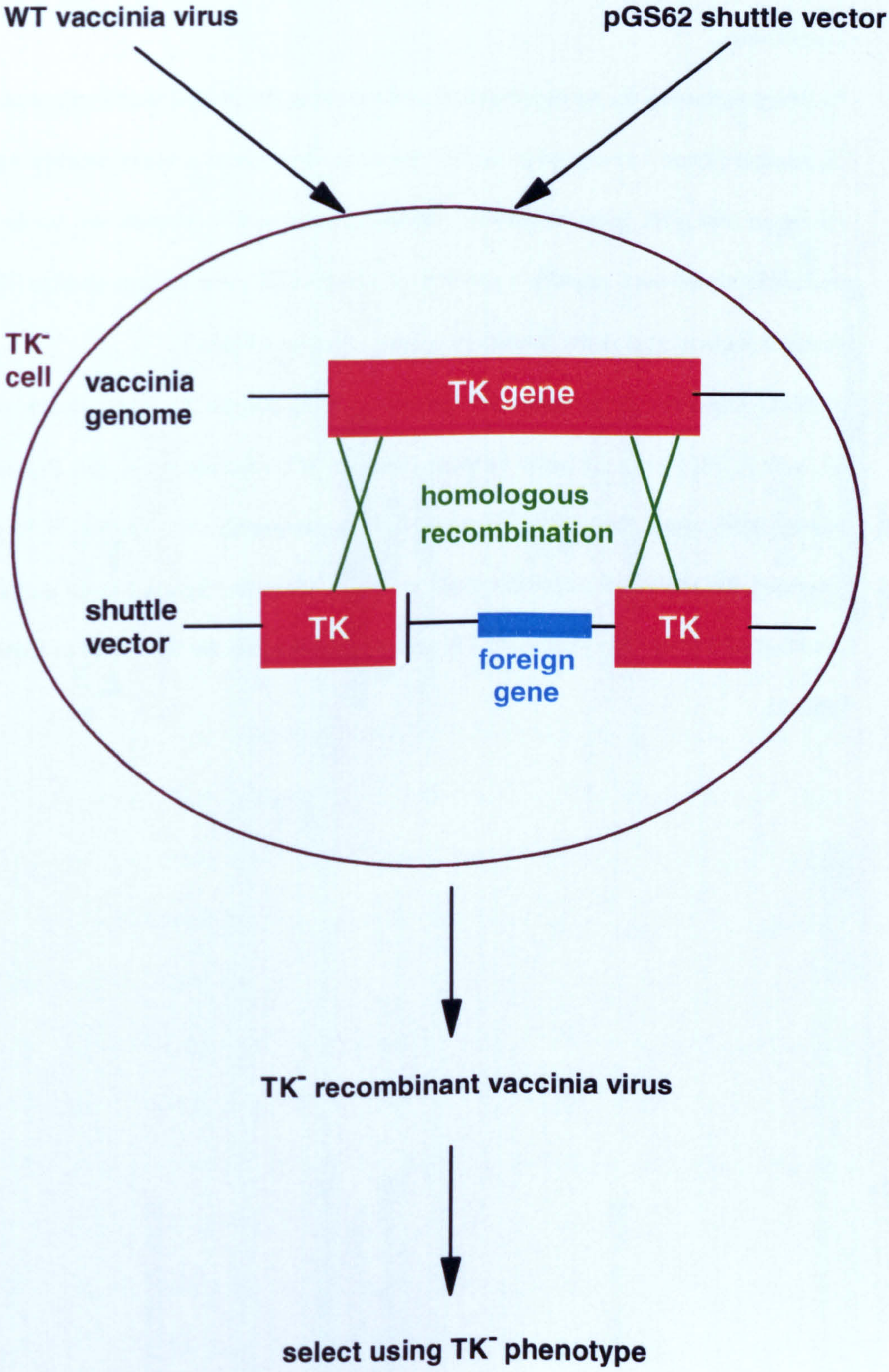

WT vaccinia virus
pGS62 shuttle vector
TK⁻ cell
vaccinia genome
TK gene
homologous recombination
shuttle vector
TK
TK
foreign gene
TK⁻ recombinant vaccinia virus
select using TK⁻ phenotype

FIGURE 16. ANALYSIS OF RECOMBINANT VACCINIA VIRUS BY PCR

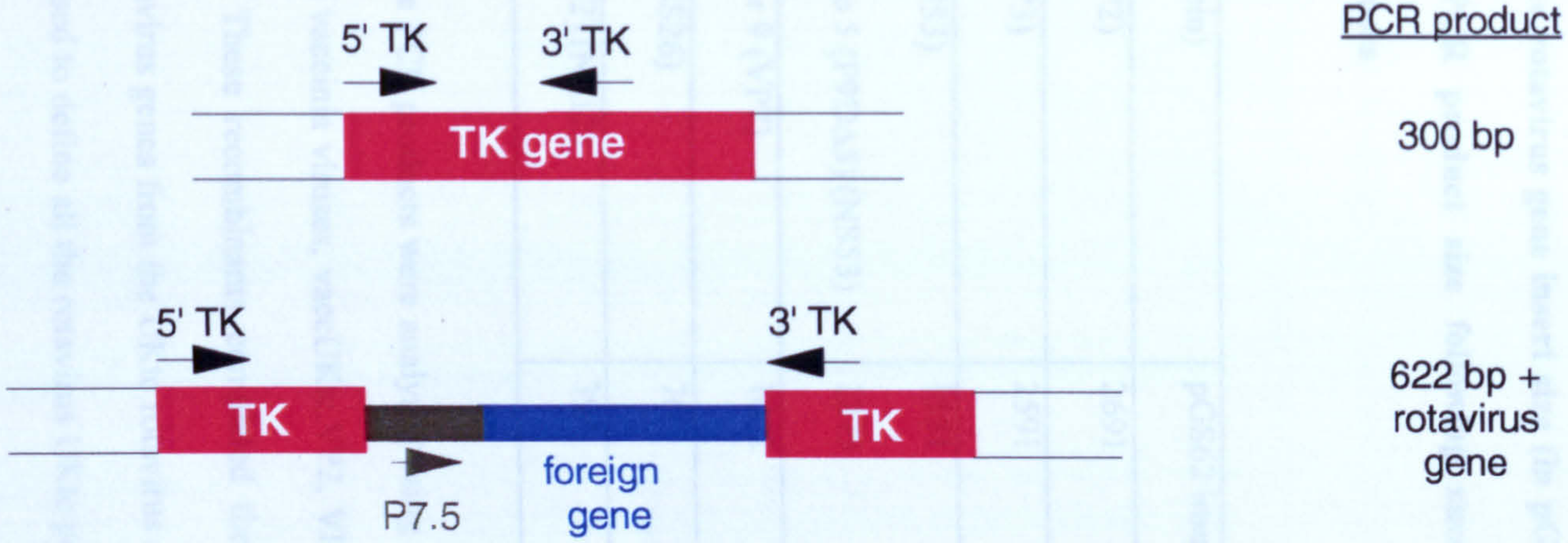
Potential recombinant vaccinia virus were screened by PCR. Two sets of primers were employed depending on the vaccinia shuttle plasmid used in the construction of the recombinants.

(A) When screening for recombinants generated using the pGS62 shuttle plasmid, 5' and 3' TK primers (Table 5) were used. In WT vaccinia virus, these primers amplify a product of 300 bp in size (TK gene sequence). Recombinant vaccinia viruses are easily identified because these primers amplify a product of 300 bp (TK gene sequence) plus 322 bp ($P_{7.5}$ promoter sequence) plus the foreign gene insert size (see Table 7).

(b) When screening for recombinants generated using the pSC11-30R.2 shuttle plasmid, 5' $P_{7.5}$ and 3' TK primers (Table 5) were used. In WT vaccinia virus, the $P_{7.5}$ promoter is located well away from the TK gene and, consequently, no visible PCR product is generated. Recombinant vaccinia viruses are easily identified because these primers amplify a product of 230 bp ($P_{7.5}$ promoter/ TK gene sequence) plus the foreign gene insert size (see Table 8).

FIGURE 16

(A)


PCR product
5' TK
3' TK
wild-type vaccinia virus
TK gene
300 bp
5' TK
3' TK
recombinant vaccinia virus
generated from pGS62
(and pGS62 +ve control)
TK
TK
P7.5
foreign
gene
622 bp +
rotavirus
gene

(B)

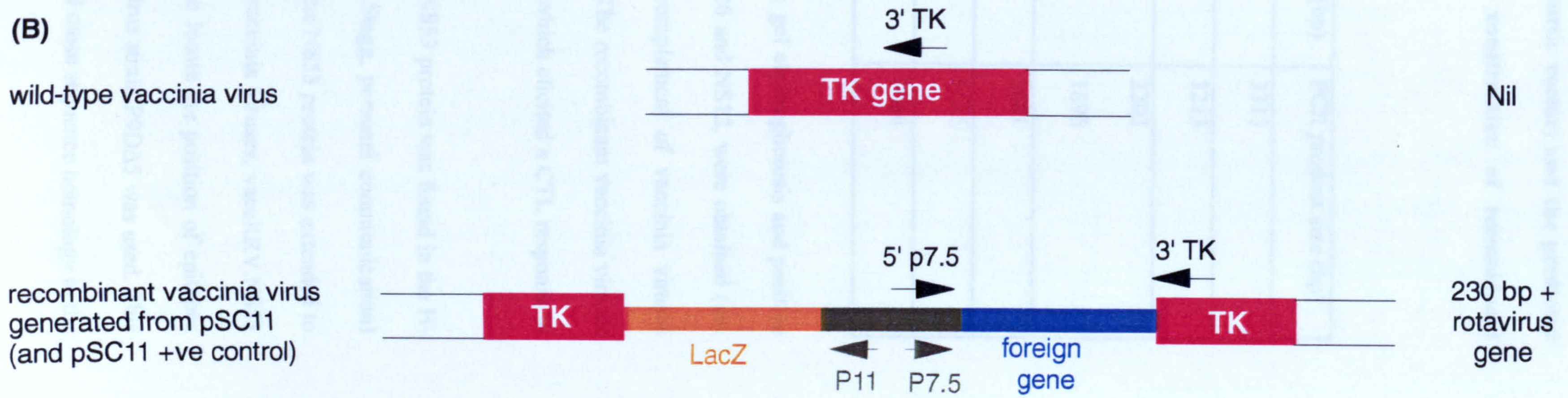

3' TK
wild-type vaccinia virus
TK gene
Nil
5' p7.5
3' TK
recombinant vaccinia virus
generated from pSC11
(and pSC11 +ve control)
TK
TK
LacZ
P11
P7.5
foreign
gene
230 bp +
rotavirus
gene

Table 7. The rotavirus gene insert size (in pGS62 shuttle vector) and the predicted amplified PCR product size following successful construction of recombinant vaccinia viruses

Gene (protein)	pGS62 insert size (bp)	PCR product size (bp)
Gene 2 (VP2)	2691	3313
Gene 3 (VP3)	2591	3213
Gene 5 (NS53) mutant gene 5 (P9DΔ5) (NS53)	1581 1273	2203 1895
Gene 7, 8 or 9 (VP7)	1062	1681
Gene 11 (NS26)	703	1325
Gene11(orf2) (NS12)	394	1016

The PCR products were analysed using agarose gel electrophoresis and positive recombinant vaccinia viruses, vaccUKtc.VP2, VP3, NS26 and NS12, were obtained (see Figure 17). These recombinants completed the full complement of vaccinia viruses carrying rotavirus genes from the UKtc rotavirus strain. The recombinant vaccinia viruses were to be used to define all the rotavirus UKtc proteins which elicited a CTL response in three different mouse haplotypes.

A specific CTL response to the rotavirus UKtc NS53 protein was found in the H-2^b mouse haplotype during the course of this study (S. Stagg, personal communication) and, for this reason, the analysis of the CTL response to the NS53 protein was extended to cover two more rotavirus strains. Positive recombinant vaccinia viruses, vaccRRV.NS53 and Hochi.NS53, were obtained (see Figure 18). Also, to locate the position of epitopes recognised by CTLs in the UKtc NS53 protein, the rotavirus strain P9DΔ5 was used. This strain was isolated in Scotland and sequence data revealed close sequence homology to the

FIGURE 17. SCREENING BY PCR OF POTENTIAL RECOMBINANT VACCINIA VIRUSES CONTAINING VARIOUS ROTAVIRUS UKTC GENES

Following transfection, potential recombinant vaccinia virus was recovered from plaques after selection in the presence of BrdU. The virus was used to infect cells and DNA samples were extracted from these as described in Materials and Methods. The vaccinia virus DNA samples were screened using PCR amplification as described in section 4.3.15 of Materials and Methods. 5' and 3' TK primers (Table 5) were used and the amplified products analysed using a 1% agarose gel. In all panels, lane M shows the DNA size markers and lane A is the Adenovirus PCR control (see Figure 14).

Panel A: Lanes 1 to 5 show screening of potential recombinant vaccinia virus containing rotavirus UKtc gene 2 (vaccUKtc.VP2). The PCR amplification gave a product of 3313 bp for positive recombinant vaccinia virus (all lanes were deemed positive).

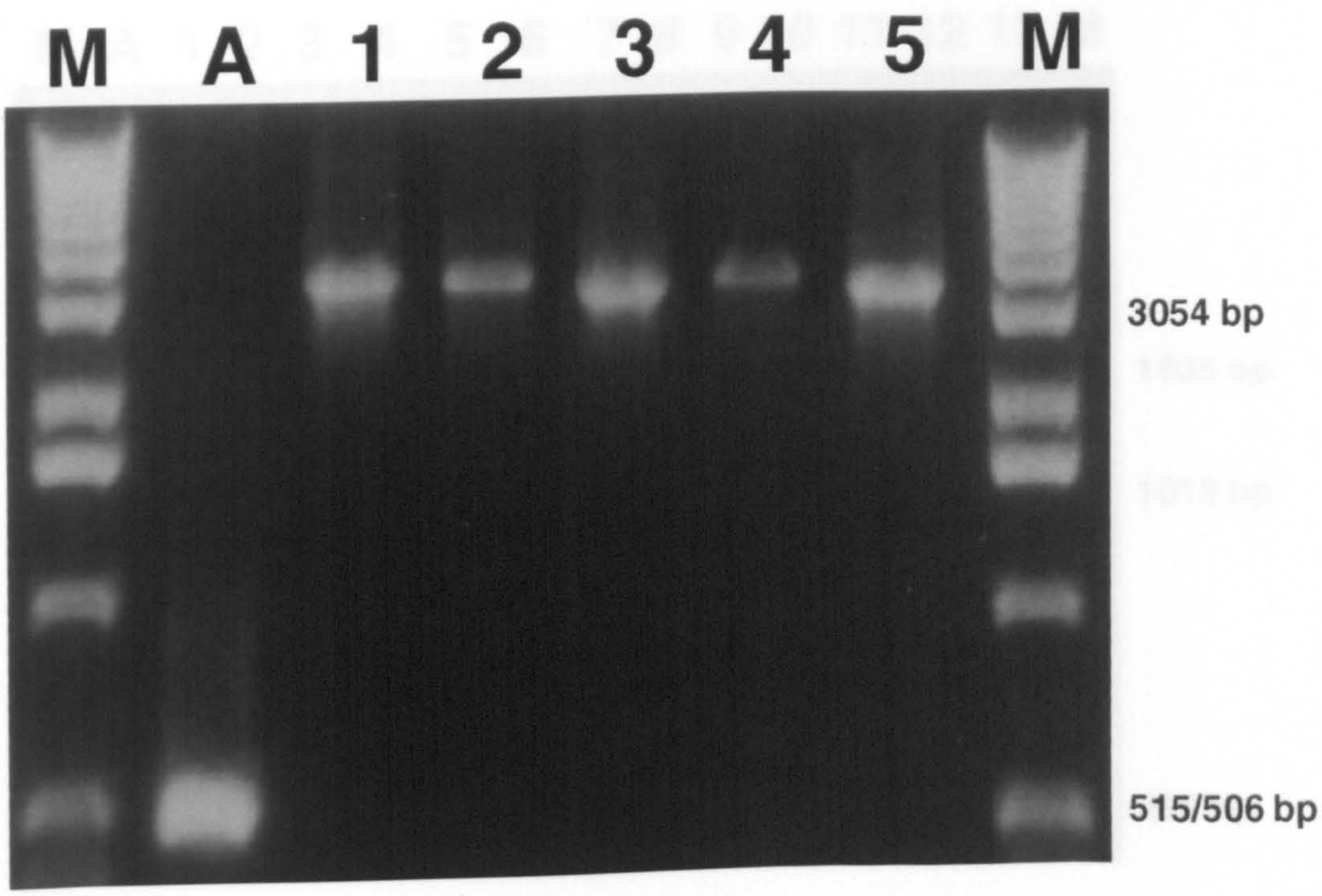
Panel B: Lanes 1 to 13 show screening of potential recombinant vaccinia virus containing rotavirus UKtc gene 3 (vaccUKtc.VP3). The PCR amplification gave a product of 3213 bp for positive recombinant vaccinia virus (lanes 1 to 3 and 5 to 13 were deemed positive).

Panel C: Lanes 1 to 13 show screening of potential recombinant vaccinia virus containing rotavirus UKtc gene 11 (vaccUKtc.NS26). The PCR amplification gave a product of 1325 bp for positive recombinant vaccinia virus (lanes 4, 5, 8 and 11 were deemed positive) and 300 bp for WT vaccinia virus.

Panel D: Lanes 1 to 10 show screening of potential recombinant vaccinia virus containing rotavirus UKtc gene 11(orf2) (vaccUKtc.NS12). The PCR amplification gave a product of 1016 bp for positive recombinant vaccinia virus (lanes 4 and 7 were deemed positive) and 300 bp for WT vaccinia virus.

FIGURE 17

(A)


M A 1 2 3 4 5 M
3054 bp
515/506 bp

(B)

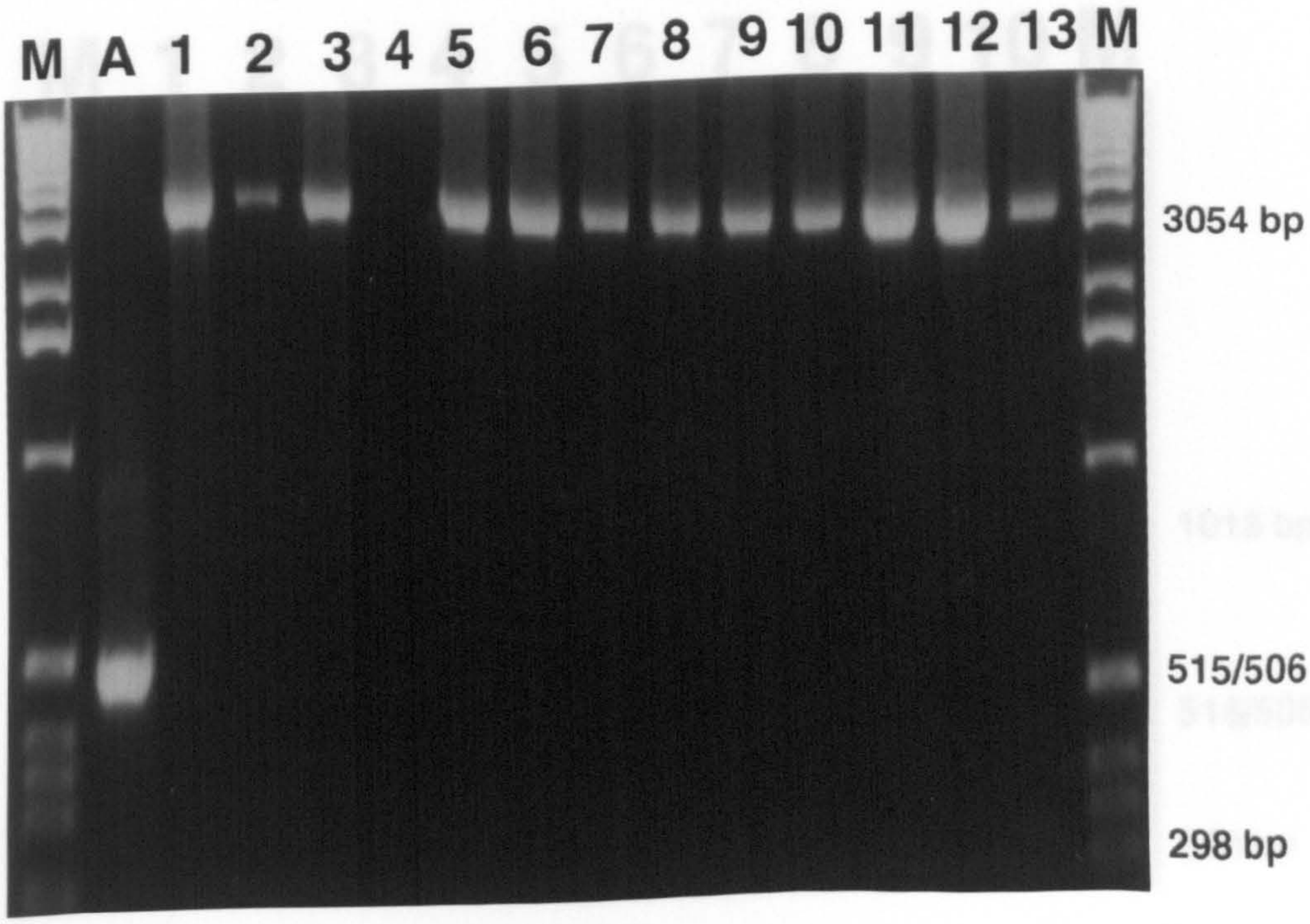
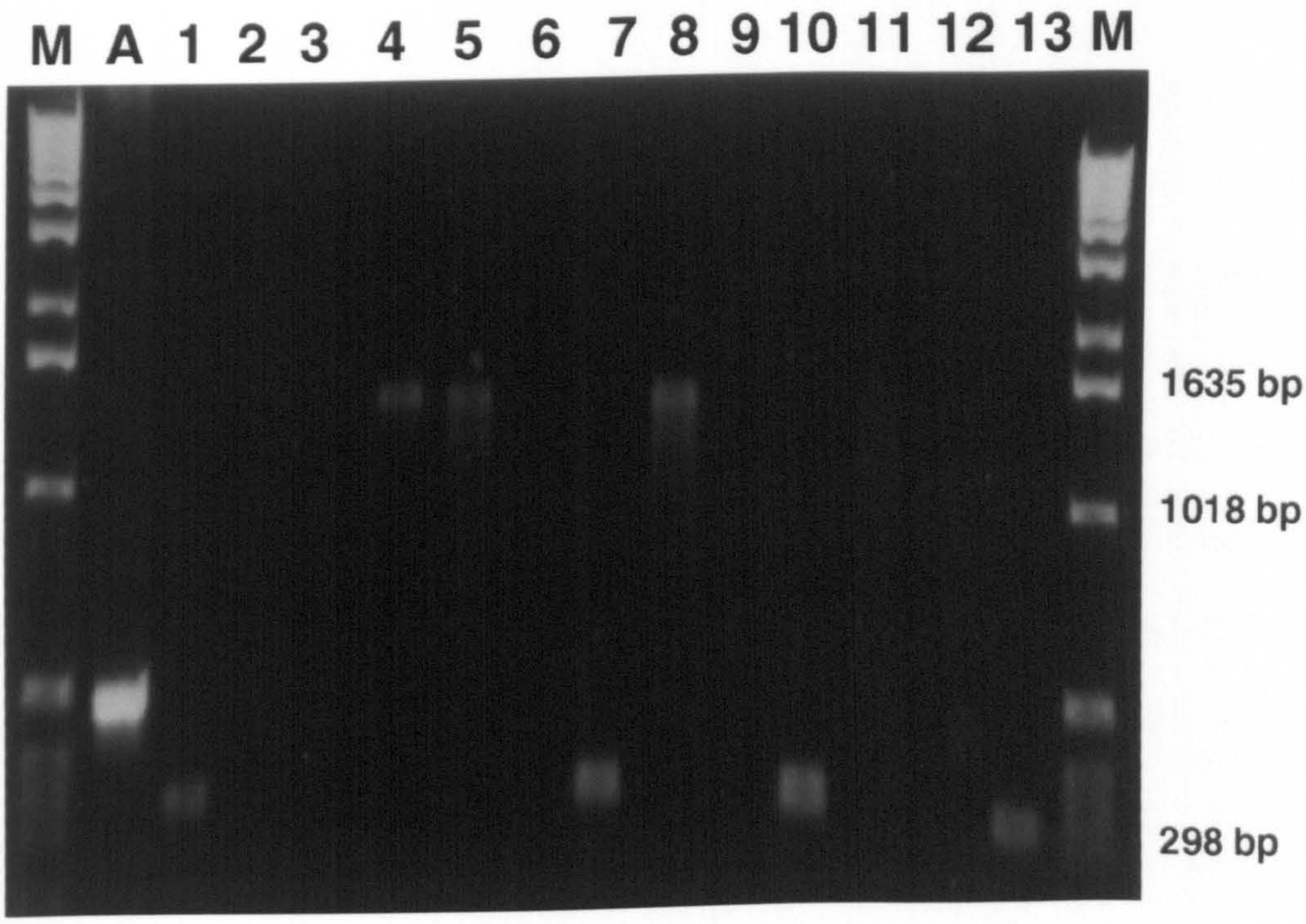

M A 1 2 3 4 5 6 7 8 9 10 11 12 13 M
3054 bp
515/506 bp
298 bp

FIGURE 17

(C)


M A 1 2 3 4 5 6 7 8 9 10 11 12 13 M
1635 bp
1018 bp
298 bp

(D)

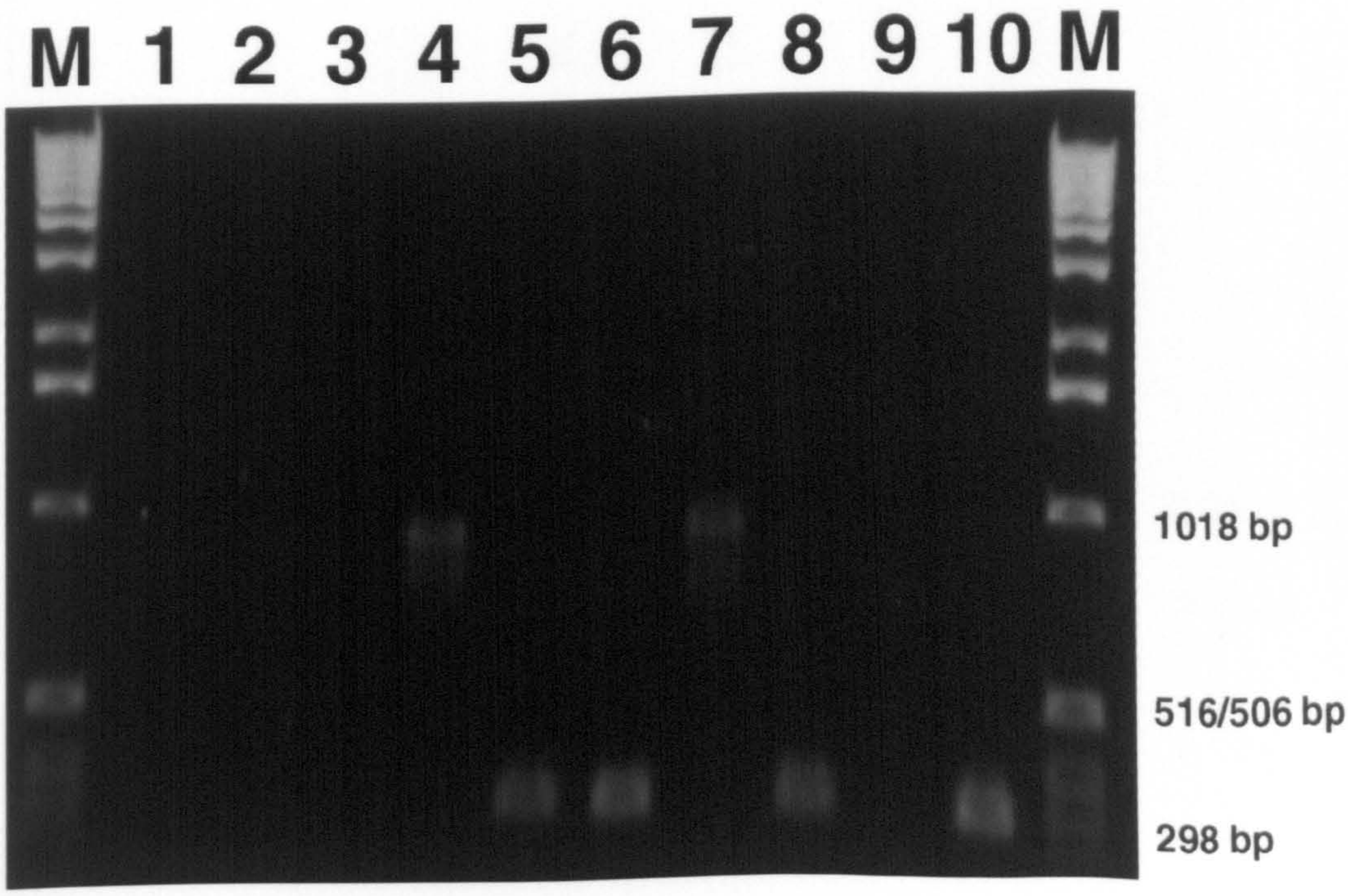

M 1 2 3 4 5 6 7 8 9 10 M
1018 bp
516/506 bp
298 bp

FIGURE 18. SCREENING BY PCR OF POTENTIAL RECOMBINANT VACCINIA VIRUSES CONTAINING GENE 5 FROM VARIOUS ROTAVIRUS STRAINS

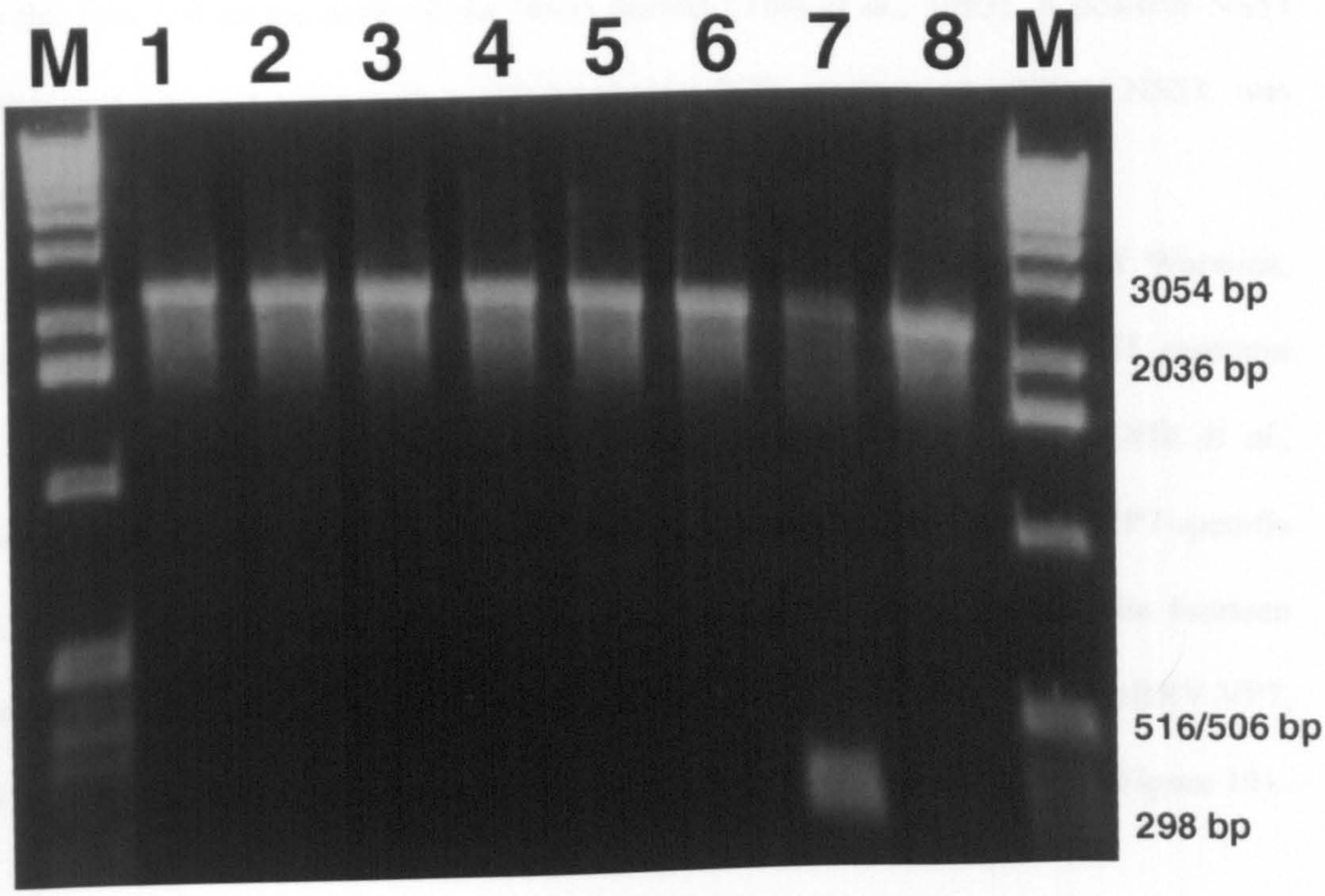
The PCR amplification and subsequent analysis of the products was carried out as detailed in Figure 17 again using the 5' and 3' TK primers. In both panels, lane M shows the DNA size markers.

Panel A: Lanes 1 to 8 show screening of potential recombinant vaccinia virus containing rotavirus RRV gene 5 (vaccRRV.NS53). The PCR amplification gave a product of 2203 bp for positive recombinant vaccinia virus (all lanes were deemed positive) and 300 bp for WT vaccinia virus (lane 7 appears to be mixed plaque containing both recombinant vaccinia virus and WT vaccinia virus).

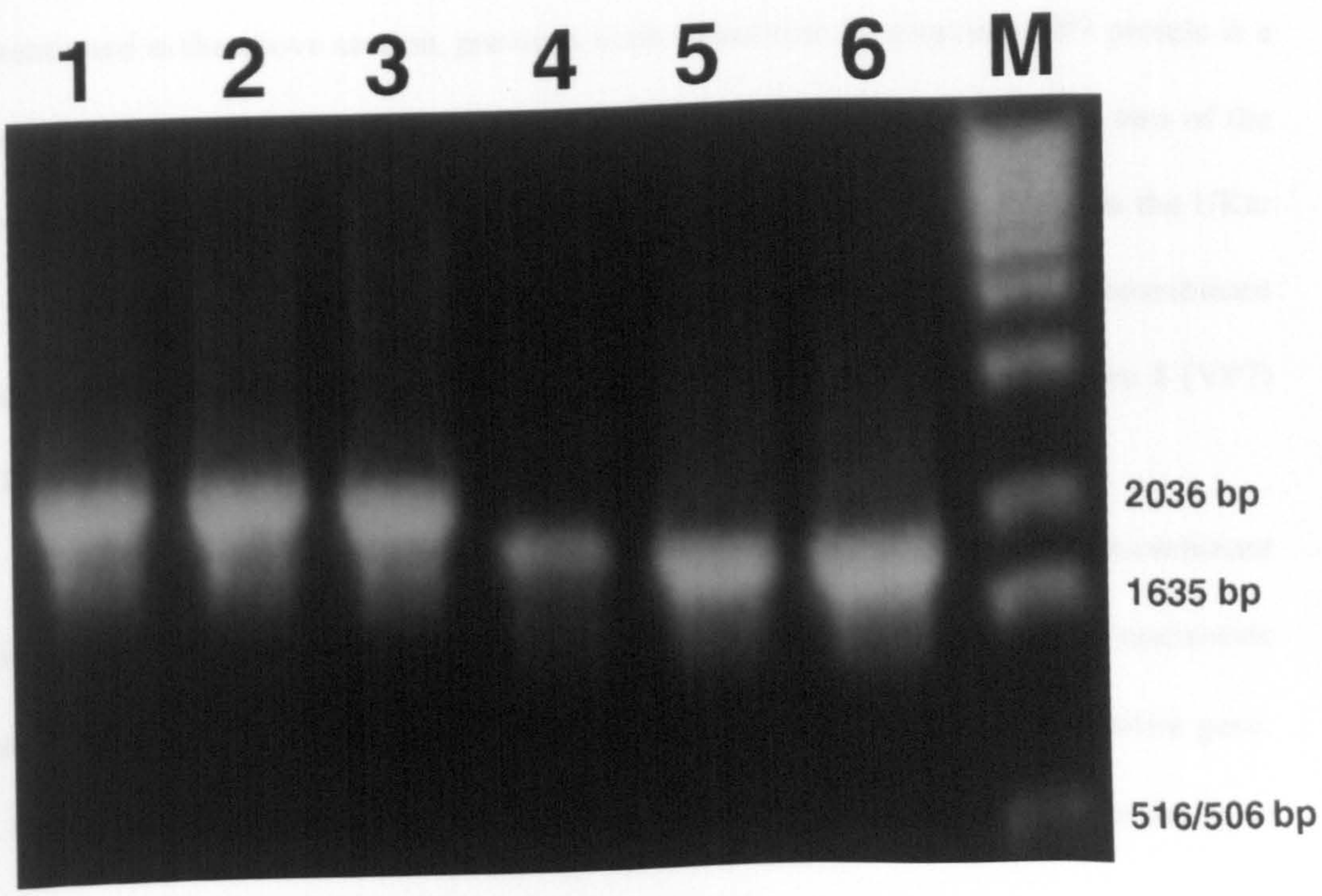
Panel B: Lanes 1 to 3 show screening of potential recombinant vaccinia virus containing rotavirus Hochi gene 5 (vaccHochi.NS53) and lanes 4 to 6 show potential recombinant vaccinia virus containing rotavirus P9DΔ5 gene 5 (vaccP9DΔ5.NS53). The PCR amplification gave a product of 2203 bp for Hochi gene 5 recombinant vaccinia virus (all lanes were deemed positive) and 1895 bp for P9DΔ5 gene 5 recombinant vaccinia virus (all lanes were deemed positive).

FIRURE 18

(A)


M 1 2 3 4 5 6 7 8 M
3054 bp
2036 bp
516/506 bp
298 bp

(B)


1 2 3 4 5 6 M
2036 bp
1635 bp
516/506 bp

UKtc strain gene 5 (Tian *et al.*, 1993). However, the P9DΔ5 gene 5 has a 300 bp deletion starting at nucleotide 450. The deletion is followed by an 'out of frame' translation stop codon which becomes 'in frame' due to the deletion and this leads to the expression of only the first 150 amino acids of the NS53 protein (Tian *et al.*, 1993). A positive NS53 recombinant vaccinia virus with a 300 bp shorter PCR product, vaccP9DΔ5.NS53, was obtained (see Figure 18).

Previous work, involving the rotavirus group at the University of Warwick, showed that the outer shell glycoprotein VP7 is a major target antigen for a CTL response that is serotype-independent between two of the fourteen VP7 serotypes (Offit *et al.*, 1994). Therefore, to extend and confirm the serotype independence of the VP7-specific CTL response, recombinant vaccinia viruses containing VP7 from seven of the fourteen serotypes were constructed. Positive recombinant vaccinia viruses, vaccRRV.VP7, A64.VP7, Hochi.VP7, 69M.VP7, Wa.VP7 and WI-61.VP7, were obtained (see Figure 19).

6.3 PRODUCTION OF RECOMBINANT VACCINIA VIRUSES CONTAINING FRAGMENTS OF ROTAVIRUS GENES (USING THE pSC11-30R.2 VECTOR)

As mentioned in the above section, previous work found that the rotavirus VP7 protein is a major target antigen for a CTL response that is serotype-independent between two of the fourteen VP7 serotypes (Offit *et al.*, 1994). To locate the position of epitopes in the UKtc VP7 protein recognised by CTLs, F. Xu began the construction of a panel of recombinant vaccinia viruses containing restriction enzyme digested fragments of UKtc gene 8 (VP7) (see Figure 20).

Previous work at Warwick had shown that a rotavirus UKtc VP7 recombinant vaccinia virus which did not containing the 5' end of the gene to the ClaI site at nucleotide 90 gave the same CTL response as a recombinant containing a cDNA of the entire gene. The UKtc gene 8 also contains two in-frame ATG's used for translation initiation at

FIGURE 19. SCREENING BY PCR OF POTENTIAL RECOMBINANT VACCINIA VIRUSES CONTAINING THE GENE (7, 8 OR 9) ENCODING THE VP7 PROTEIN OF VARIOUS ROTAVIRUS STRAINS

The PCR amplification and subsequent analysis of the products was carried out as detailed in Figure 17 using the 5' and 3' TK primers. In all panels, lane M shows the DNA size markers and lane A shows the Adenovirus PCR control (see Figure 14). The PCR amplification gave a product of 1681 bp for gene 9 recombinant vaccinia virus and 300 bp for WT vaccinia virus.

Panel A: Lanes 1 to 11 show screening of potential recombinant vaccinia virus containing rotavirus RRV gene 9 (vaccRRV.VP7). Lanes 1, 2, 7, 8, 10 and 11 were deemed positive.

Panel B: Lanes 1 to 11 show screening of potential recombinant vaccinia virus containing rotavirus A64 gene 9 (vaccA64.VP7). Lanes 1 and 7 were deemed positive.

Panel C: Lanes 1 to 9 show screening of potential recombinant vaccinia virus containing rotavirus Hochi gene 9 (vaccHochi.VP7). Lane 3 was deemed positive.

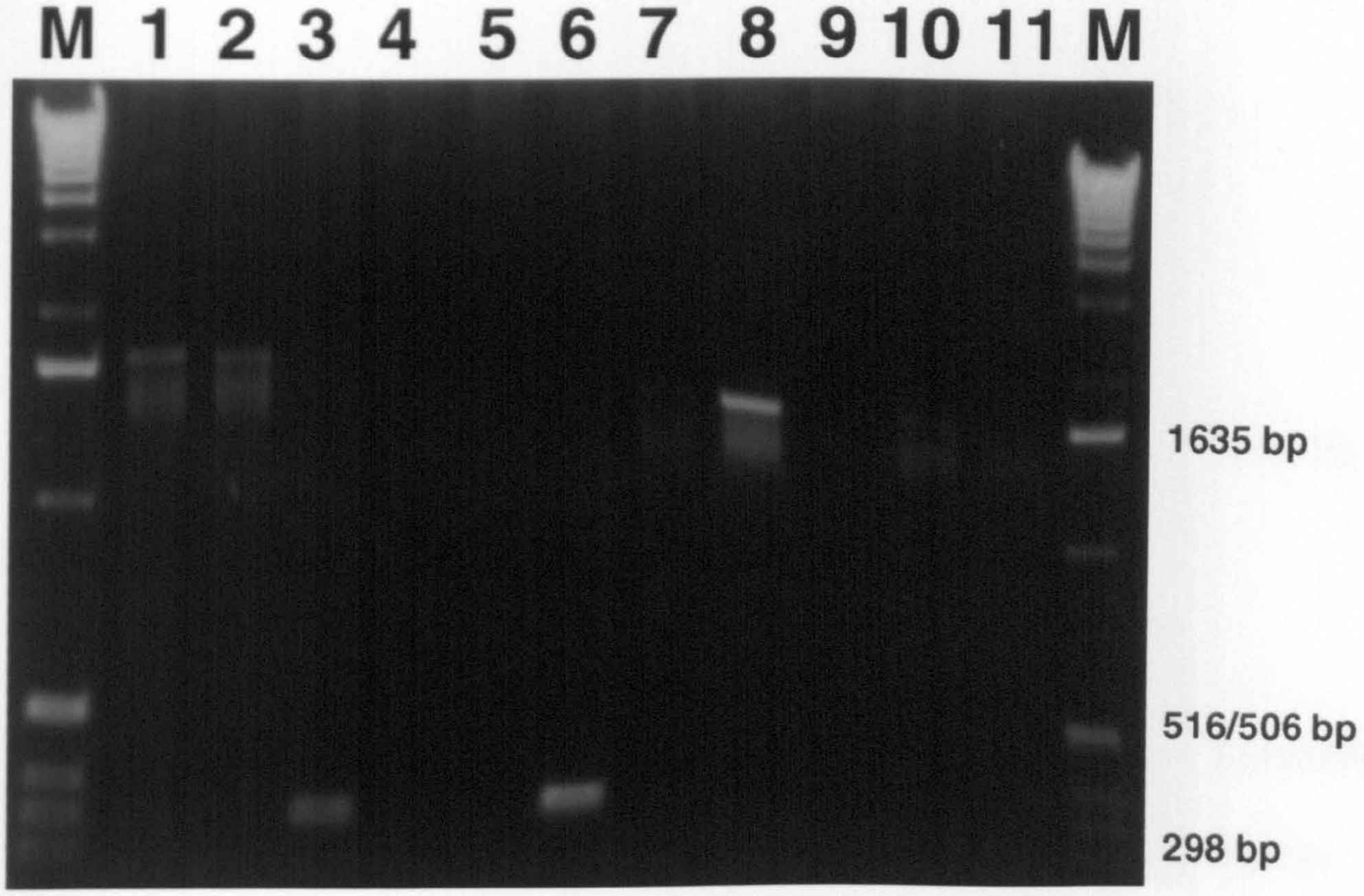
Panel D: Lanes 1 to 11 show screening of potential recombinant vaccinia virus containing rotavirus 69M gene 9 (vacc69M.VP7). Lanes 1, 3, 4, 5 and 9 were deemed positive.

Panel E: Lanes 1 to 11 show screening of potential recombinant vaccinia virus containing rotavirus Wa gene 9 (vaccWa.VP7). Lanes 3, 5 and 9 were deemed positive.

Panel F: Lanes 1 to 11 show screening of potential recombinant vaccinia virus containing rotavirus WI-61 gene 9 (vaccWI-61.VP7). Lanes 1, 2, 3, 8, 9 and 10 were deemed positive.

FIGURE 19

(A)


M 1 2 3 4 5 6 7 8 9 10 11 M
1635 bp
516/506 bp
298 bp

(B)

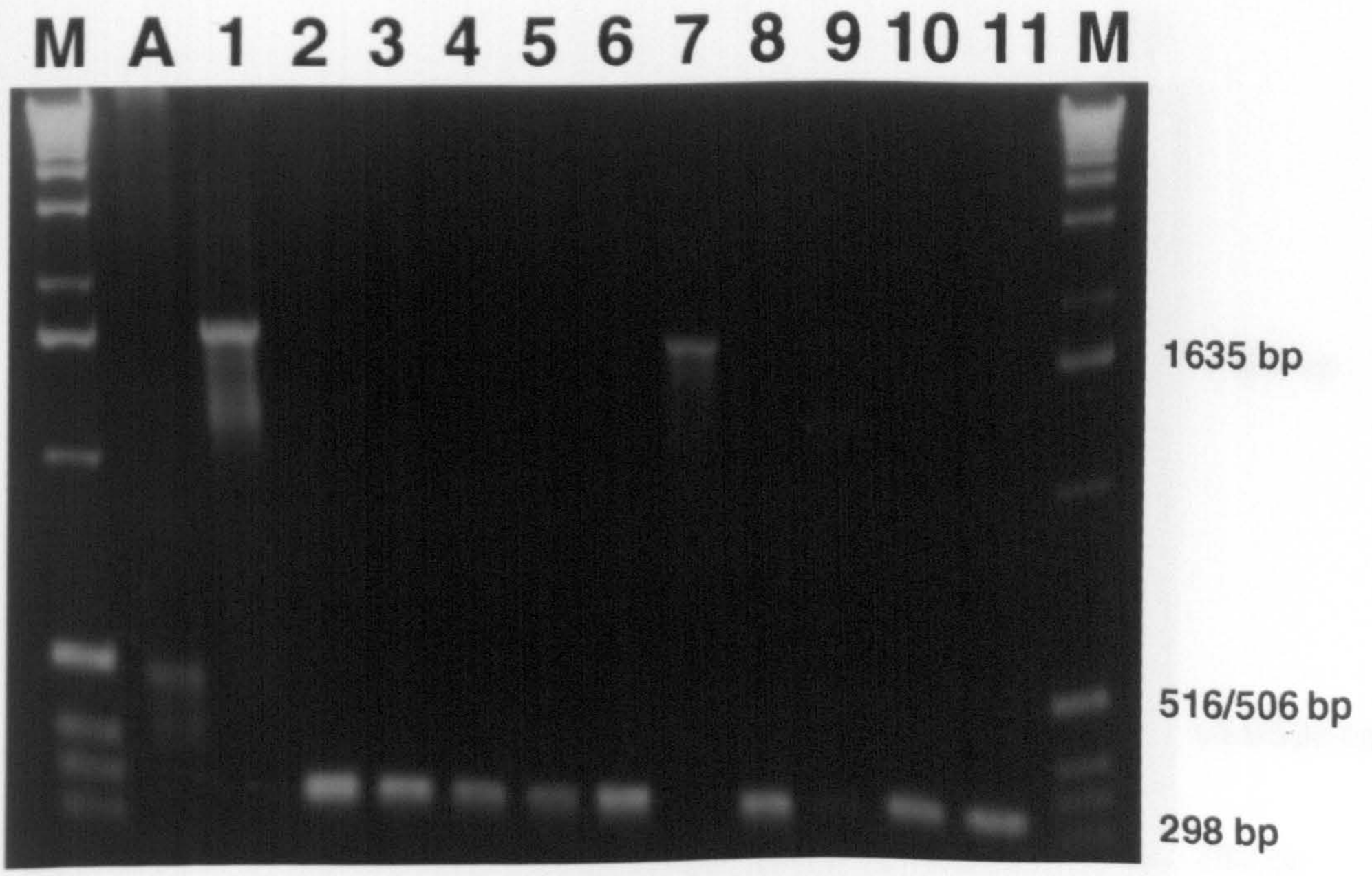
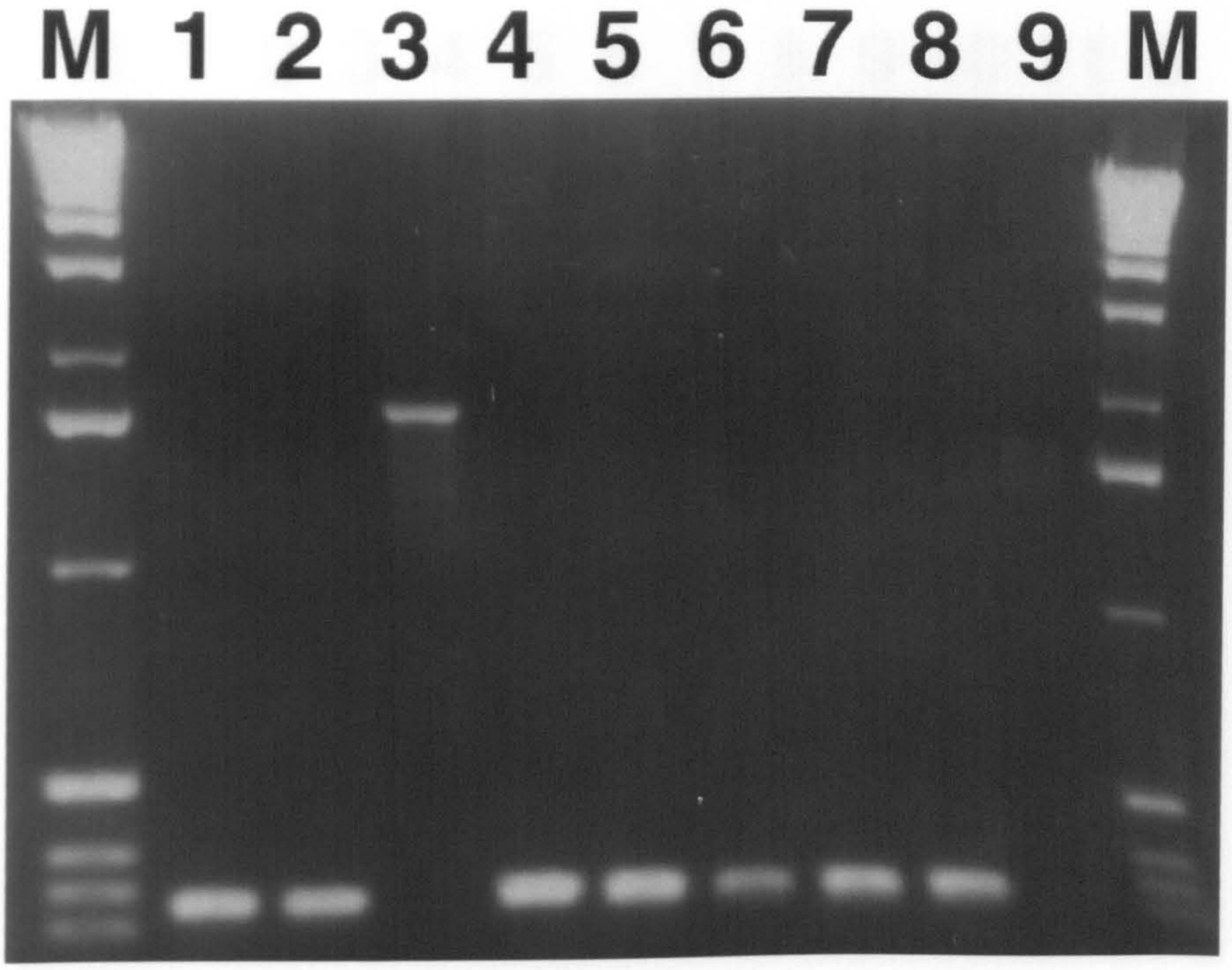

M A 1 2 3 4 5 6 7 8 9 10 11 M
1635 bp
516/506 bp
298 bp

FIGURE 19

(C)


M 1 2 3 4 5 6 7 8 9 M
1635 bp
515/506 bp
298 bp

(D)

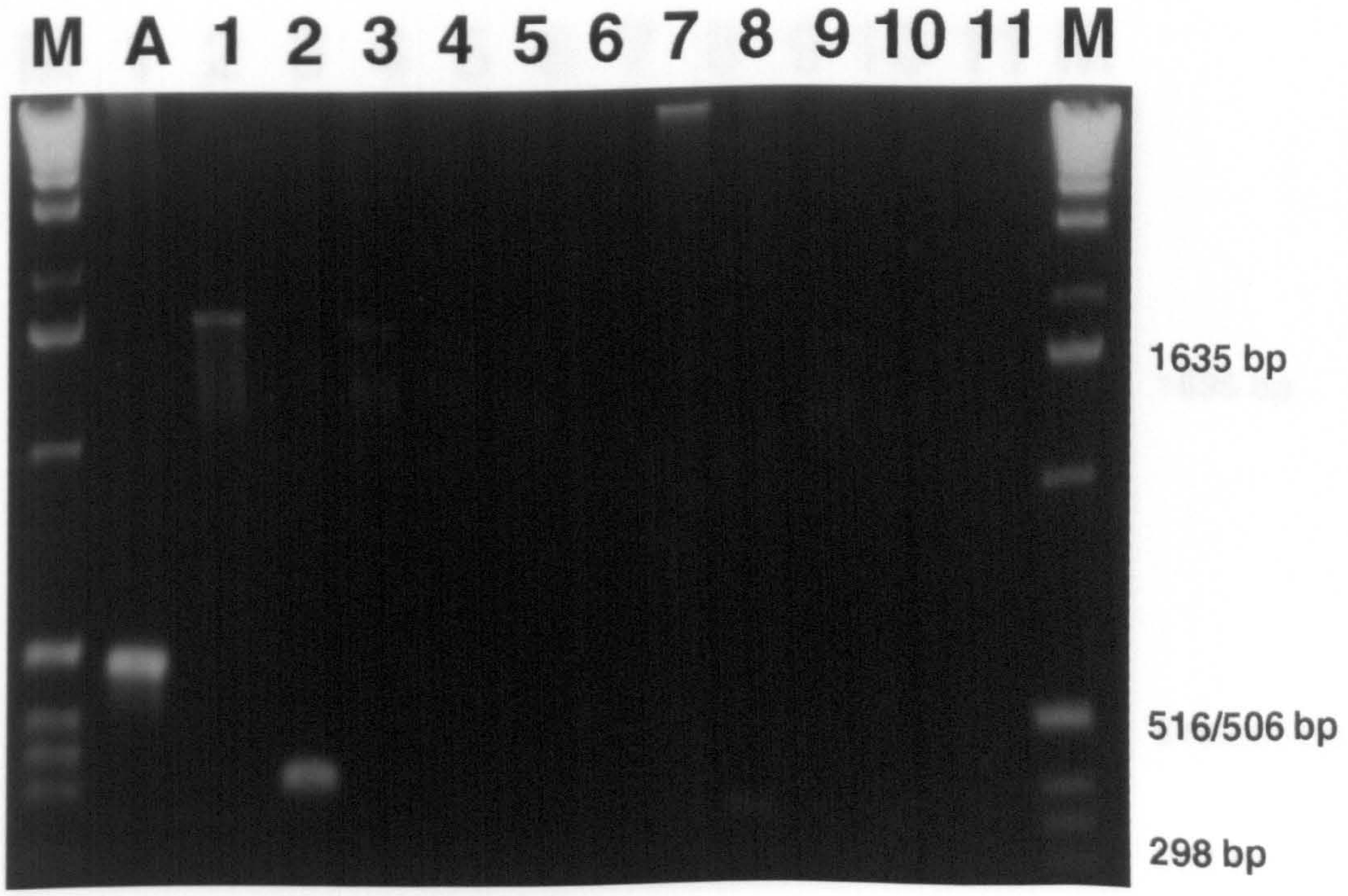
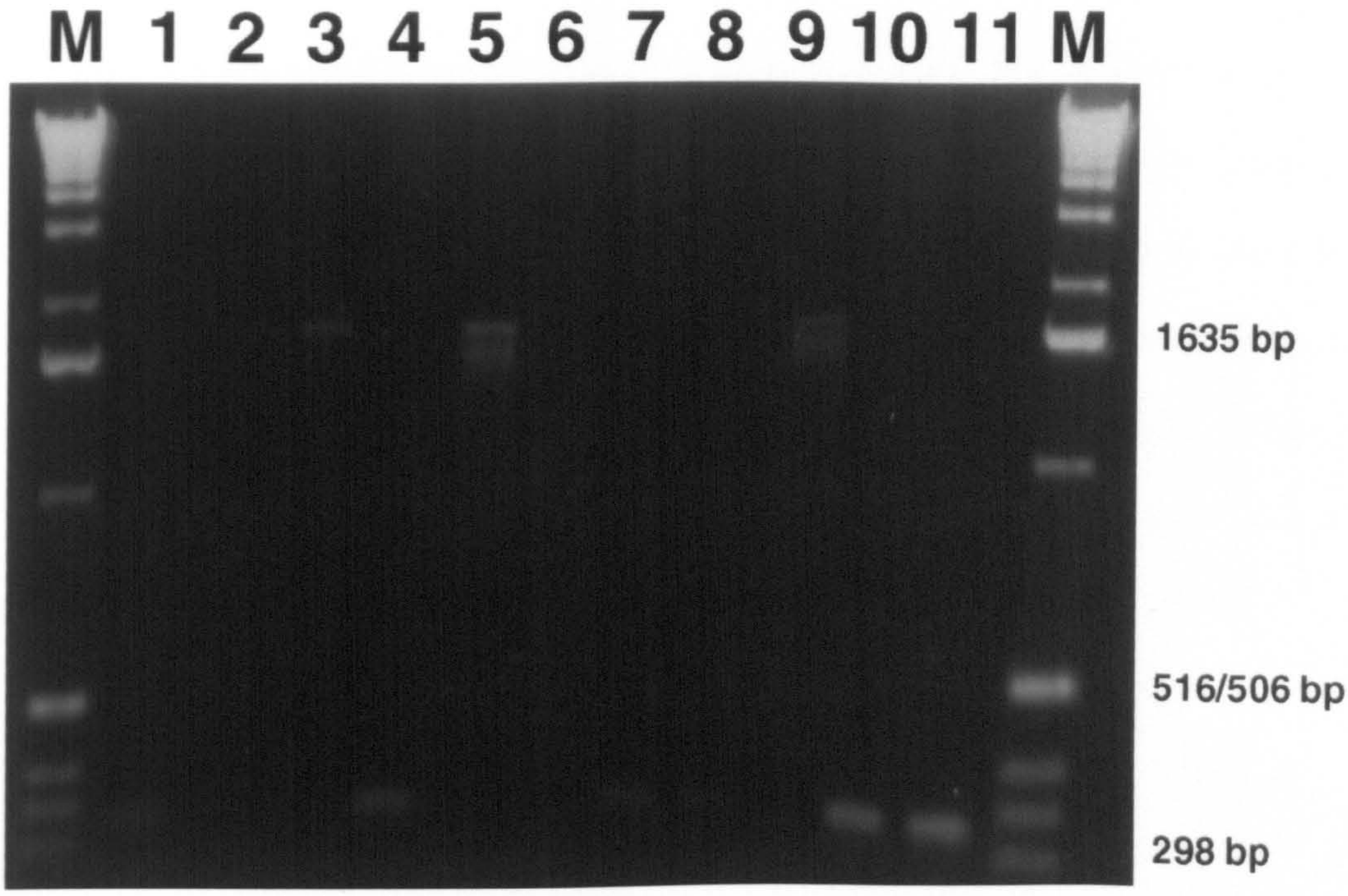

M A 1 2 3 4 5 6 7 8 9 10 11 M
1635 bp
516/506 bp
298 bp

FIGURE 19

(E)


M 1 2 3 4 5 6 7 8 9 10 11 M
1635 bp
516/506 bp
298 bp

(F)

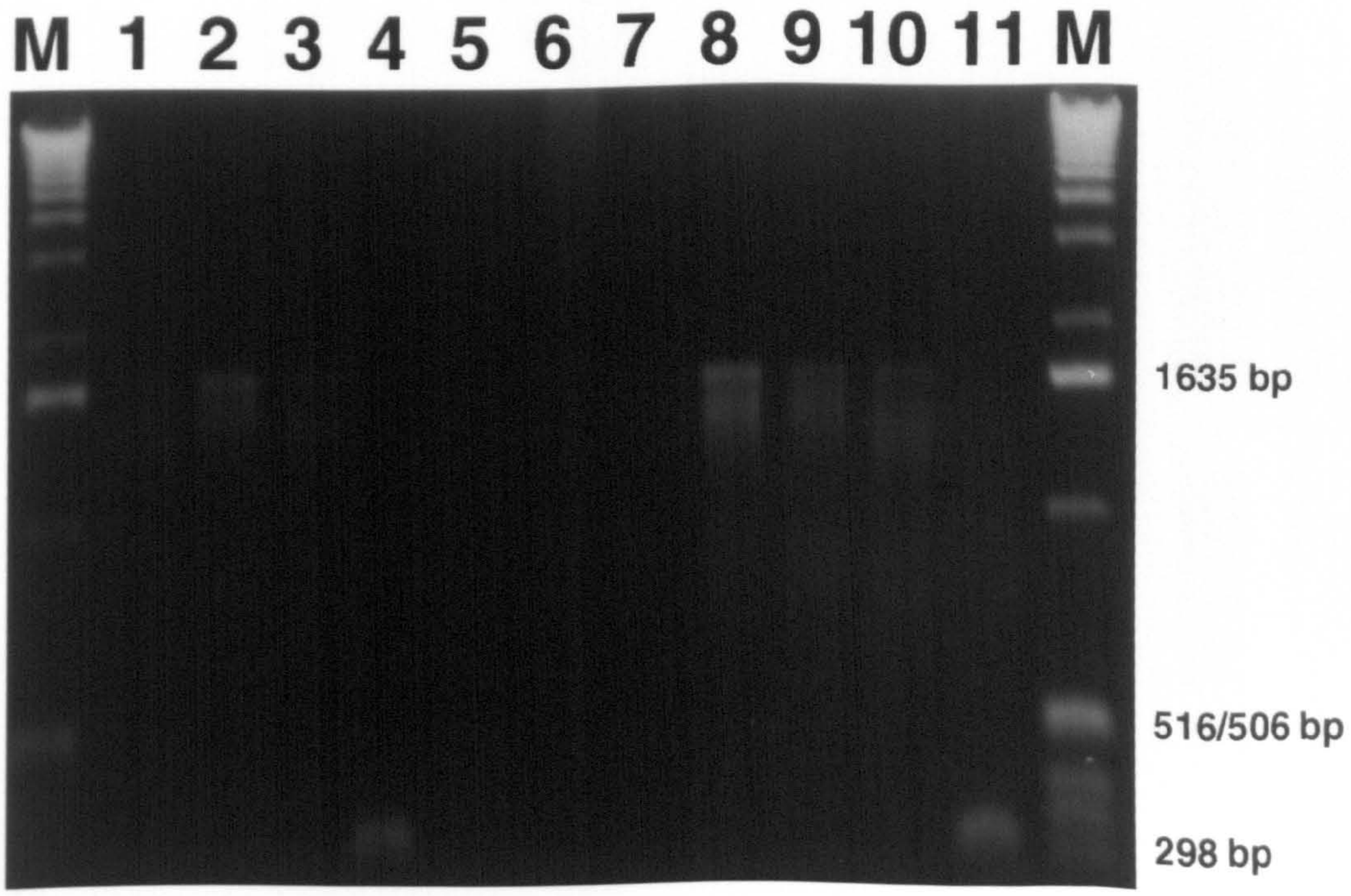
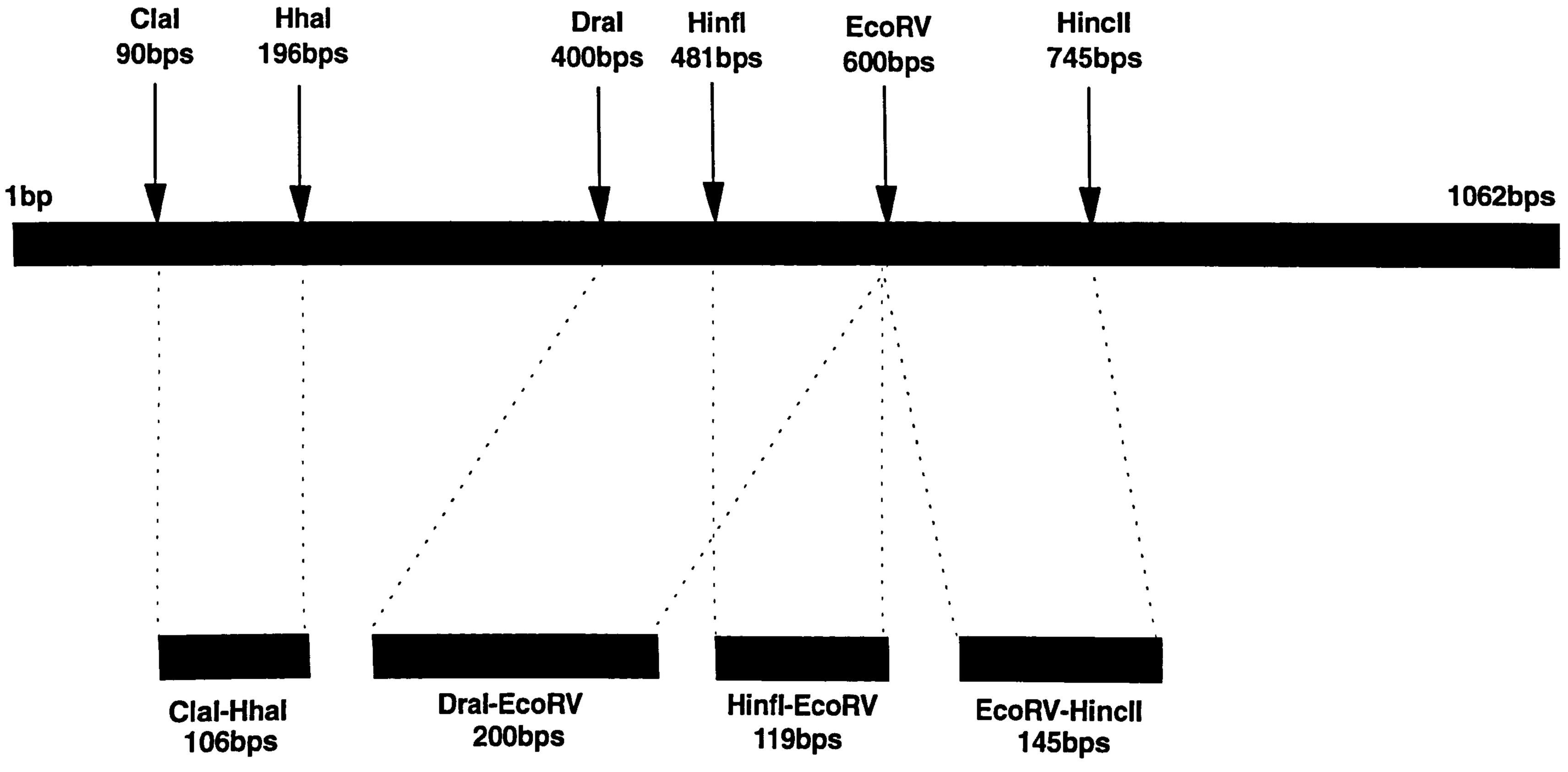

M 1 2 3 4 5 6 7 8 9 10 11 M
1635 bp
516/506 bp
298 bp

FIGURE 20. UKTC GENE 8 (VP7 PROTEIN) FRAGMENTS CLONED INTO pSC11-30R.2

The fragments of rotavirus UKtc gene 8 were derived from a full-length cDNA inserted into the Bluescribe cloning vector. F. Xu digested UKtc gene 8 with a number of restriction enzymes to produce four fragments. After purification using the Gene Clean® II kit, the fragments were rendered 'blunt-ended' before insertion into the pSC11-30R.2 vaccinia shuttle vector (see Figure 21). The vector was digested with the appropriate enzyme to allow 'in-frame' insertion. The fragments ClaI-HhaI and HinfI-EcoRV were received cloned into pSC11-30R.2 whereas, DraI-EcoRV and EcoRV-HincII were received as recombinant vaccinia viruses.

FIGURE 20


ClaI
90bps
HhaI
196bps
DraI
400bps
HinfI
481bps
EcoRV
600bps
HincII
745bps
1bp
1062bps
ClaI-HhaI
106bps
DraI-EcoRV
200bps
HinfI-EcoRV
119bps
EcoRV-HincII
145bps

nucleotides 49 and 139 respectively, the second having a stronger consensus sequence for initiation. For these reasons, F. Xu concentrated on cloning fragments of UKtc gene 8 spanning from the ClaI site to the 3' end of this gene. During the period of her study, she had cloned the ClaI-HhaI, DraI-EcoRV, HinfI-EcoRV and EcoRV-HincII UKtc gene 8 fragments into the pSC11-30R.2 vaccinia shuttle vector and, in addition, constructed recombinant vaccinia viruses, vaccUKtc.VP7(DraI-EcoRV) and (EcoRV-HincII), from their corresponding shuttle vectors.

The pSC11-30R.2 vector (see Figure 21) is similar to the pGS62 vaccinia shuttle vector in that the cloned genes are linked to the vaccinia virus compound early/late $P_{7.5}$ promoter and both are flanked by components of the non-essential vaccinia virus TK gene. The pSC11-30R.2 vector was used, rather than the pGS62, because the fragments of UKtc VP7 do not contain their own ATG translation initiation codons. The pSC11-30R.2 vector contains an inserted oligonucleotide downstream of the $P_{7.5}$ promoter which has an initiator ATG followed by three restriction enzyme sites (NcoI, SmaI and StuI) to allow translation of the inserted DNA in each of the three potential reading frames. Three stop codons are also included in the inserted oligonucleotide, downstream of the insert site, to terminate translation in each of the three reading frames.

The transfection method used to construct recombinant vaccinia viruses from the pSC11-30R.2 vaccinia shuttle vector was identical to that employed for pGS62 (see section 6.2). Viruses from up to twenty plaques for each transfected UKtc gene 8 fragment, deemed TK^- following selection with BrdU, were again recovered and resuspended in medium. A fifth of each sample was propagated on CV-1 cells and the DNA extracted following complete cpe.

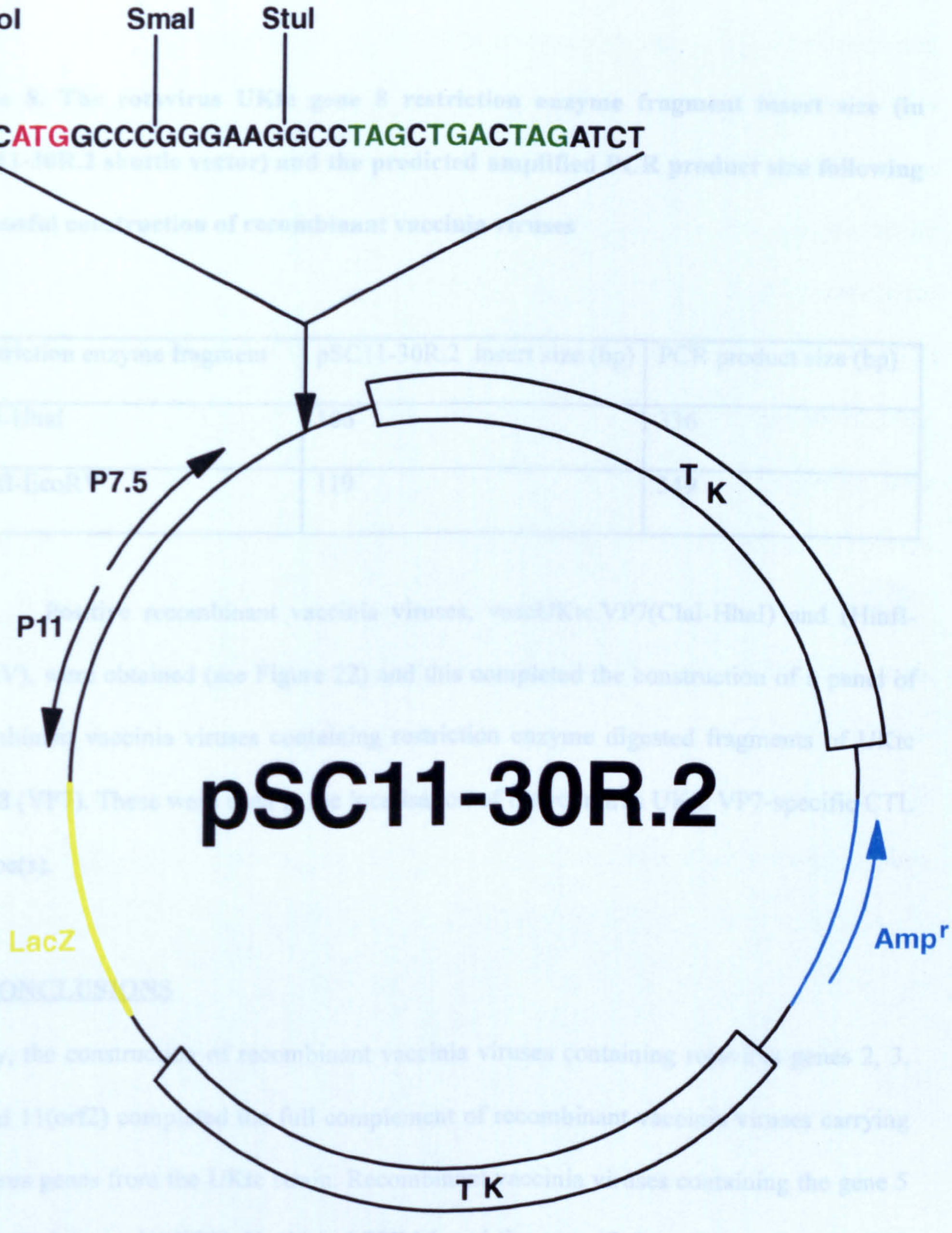
The extracted DNA was subjected to PCR amplification using the 5' $P_{7.5}$ and 3' TK primers (see Table 5). In WT vaccinia virus , the $P_{7.5}$ promoter is located well away from the TK gene and, as a consequence, no visible PCR products were generated. From

FIGURE 21. MAP OF THE SHUTTLE PLASMID VECTOR pSC11-30R.2

pSC11 is a well-known vaccinia shuttle vector (Chakrabarti *et al.* 1985). Shown in blue is the bacterial component of this plasmid containing an origin of replication for propagation in *E. coli* and an ampicillin resistance gene (Amp^r) to allow selection of bacteria carrying this plasmid. The lacZ gene of *E. coli* is shown in yellow and can be used to screen potential recombinant vaccinia virus. The vaccinia virus components (in black) consist of two promoters, P_{11} and P_{75} which drive expression of the lacZ gene and the foreign gene respectively. These elements had been placed within the vaccinia virus thymidine kinase (TK) gene which allows recombination, within infected cells, between the shuttle plasmid and the wild-type vaccinia virus genome. This generates a recombinant vaccinia virus (with a TK^- phenotype) which contains the gene of interest and control elements for its expression during virus replication.

The pSC11 vector was modified by insertion of an oligonucleotide (sequence shown) at the SmaI site to generate pSC11-30R.2. The oligonucleotide contains an initiator ATG downstream of which are three unique restriction enzymes sites (NcoI, SmaI and StuI) providing for insertion of foreign gene fragments in each reading frame and, finally, stop codons in each of the three frames.

FIGURE 21


NcoI
SmaI
StuI
CCATGGCCCGGGAAGGCCTAGCTGACTAGATCT
P7.5
T K
P11
pSC11-30R.2
LacZ
Amp r
T K

positive recombinant vaccinia viruses, these primers amplified a product including the flanking $P_{7.5}$/TK gene sequences (230 bp) plus the foreign gene insert (See Figure 16B and Table 8).

Table 8. The rotavirus UKtc gene 8 restriction enzyme fragment insert size (in pSC11-30R.2 shuttle vector) and the predicted amplified PCR product size following successful construction of recombinant vaccinia viruses

Restriction enzyme fragment	pSC11-30R.2 insert size (bp)	PCR product size (bp)
ClaI-HhaI	106	336
HinfI-EcoRV	119	349

Positive recombinant vaccinia viruses, vaccUKtc.VP7(ClaI-HhaI) and (HinfI-EcoRV), were obtained (see Figure 22) and this completed the construction of a panel of recombinant vaccinia viruses containing restriction enzyme digested fragments of UKtc gene 8 (VP7). These were used in the localisation of the rotavirus UKtc VP7-specific CTL epitope(s).

6.4 CONCLUSIONS

Firstly, the construction of recombinant vaccinia viruses containing rotavirus genes 2, 3, 11 and 11(orf2) completed the full complement of recombinant vaccinia viruses carrying rotavirus genes from the UKtc strain. Recombinant vaccinia viruses containing the gene 5 from rotavirus strains RRV, Hochi and P9DΔ5, and the gene (7, 8 or 9) encoding the VP7 protein from rotavirus strains RRV, A64, Hochi, 69M, Wa and WI-61 were also constructed. In addition, recombinant vaccinia viruses containing restriction enzyme

FIGURE 22. SCREENING BY PCR OF POTENTIAL RECOMBINANT VACCINIA VIRUSES CONTAINING VARIOUS FRAGMENTS OF UKTC GENE 8 (VP7 PROTEIN)

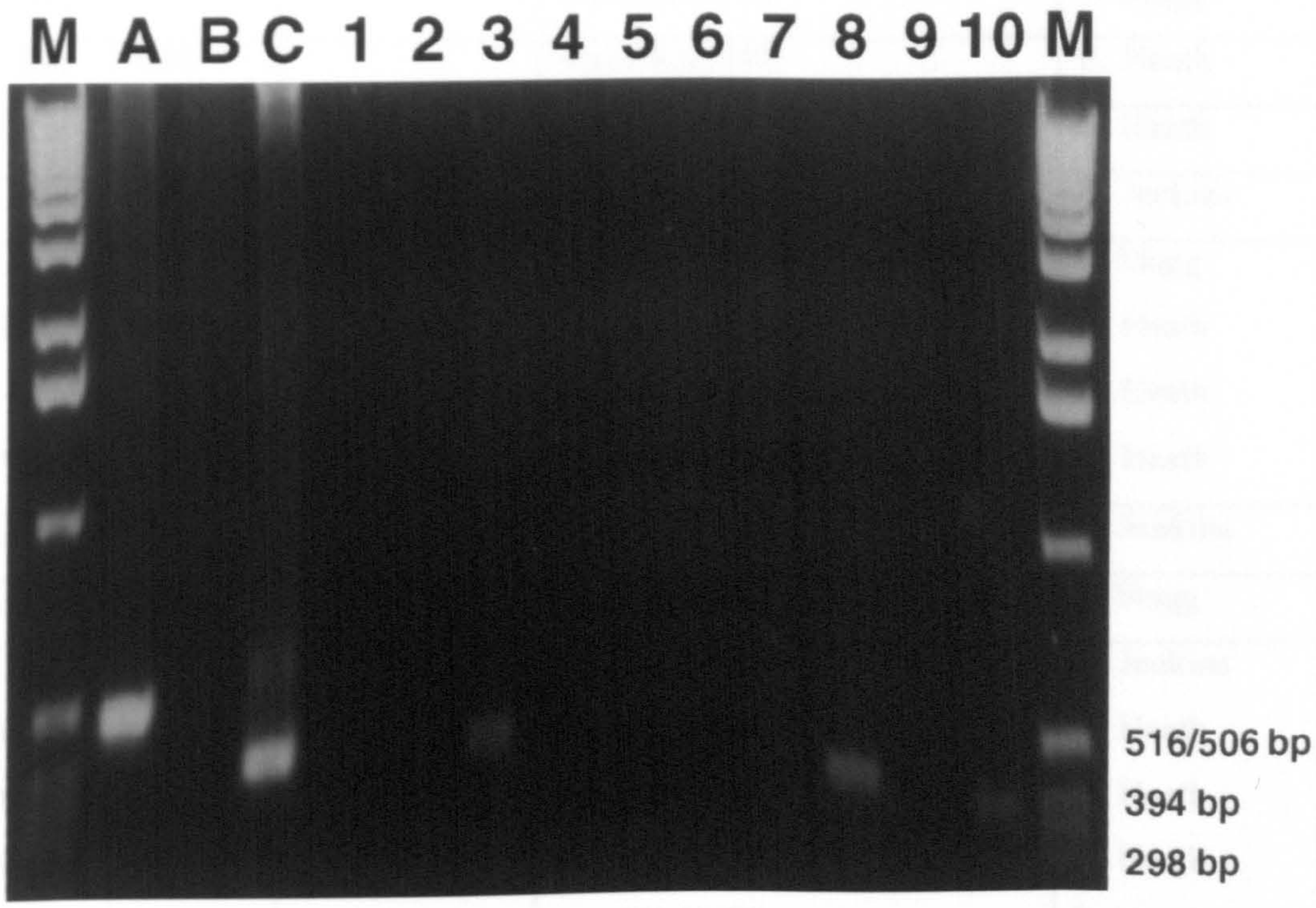
The PCR amplification and subsequent analysis of the products was carried out as detailed in Figure 17 using the 5' $P_{7.5}$ and 3' TK primers (see Table 5). In both panels, lane M shows the DNA size markers and lane A is the Adenovirus PCR control (see Figure 14).

Panel A: Lane B is a WT vaccinia virus DNA control which, due to the large distance between the $P_{7.5}$ promoter and the TK gene in the WT vaccinia virus genome, did not give a product using these PCR primers. Lane C is a PCR primer positive control for the primer pair used for the experimental samples. The primer pair was used on pSC11-30R.2 shuttle plasmid containing the ClaI-HhaI insert and the PCR amplification gave a product of 336 bp. Lanes 1 to 10 show screening of potential recombinant vaccinia virus containing the ClaI-HhaI fragment of rotavirus UKtc gene 8 [vaccUKtc.VP7(ClaI-HhaI)]. The PCR amplification gave a product of 336 bp for the positive recombinants (lanes 3, 8 and 10 were deemed positive).

Panel B: Lanes 1 to 3 show screening of potential recombinant vaccinia virus containing the HinfI-EcoRV fragment of rotavirus UKtc gene 8 [vaccUKtc.VP7(HinfI-EcoRV)]. The PCR amplification gave a product of 349 bp for positive recombinants (lane 3 was deemed positive).

FIGURE 22

(A)



(B)

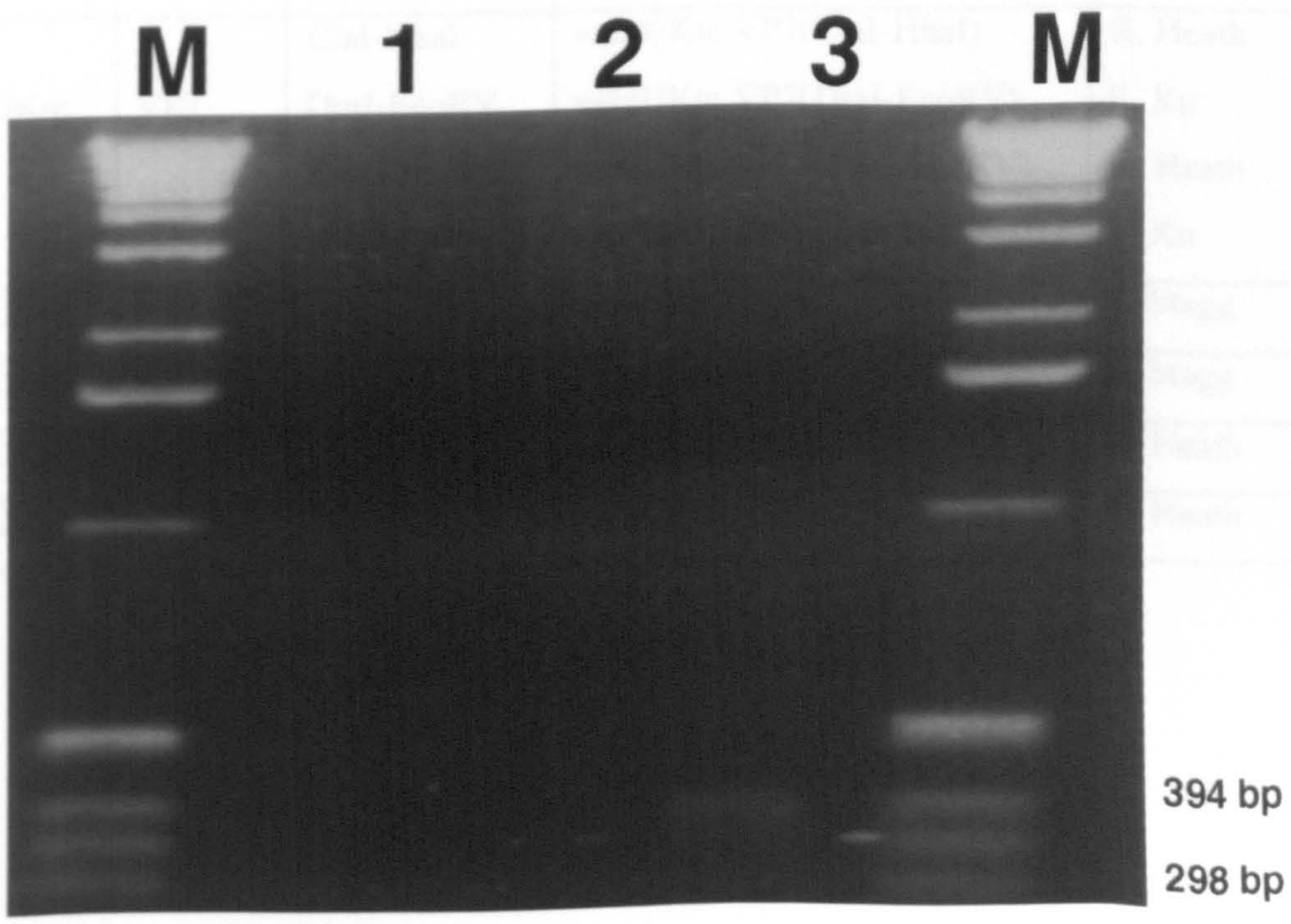


Table 9. Recombinant vaccinia viruses constructed to-date

Strain	Protein	fragment	vaccinia name	produced by
UKtc	VP1		vaccUKtc.VP1	S. Stagg
UKtc	VP2		vaccUKtc.VP2	R. Heath
UKtc	VP3		vaccUKtc.VP3	R. Heath
UKtc	VP4		vaccUKtc.VP4	R. Jenkins
Uktc RRV Hochi P9DΔ5	NS53		vaccUKtc.NS53 vaccRRV.NS53 vaccHochi.NS53 vaccP9DΔ5.NS53	S. Stagg R. Heath R. Heath R. Heath
UKtc	VP6		vaccUKtc.VP6	R. Jenkins
UKtc	NS35		vaccUKtc.NS35	S. Stagg
Uktc RRV A64 Hochi 69M Wa WI-61	VP7		vaccUKtc.VP7 vaccRRV.VP7 vaccA64.VP7 vaccHochi.VP7 vacc69M.VP7 vaccWa.VP7 vaccWI-61.VP7	R. Jenkins R. Heath R. Heath R. Heath R. Heath R. Heath R. Heath
UKtc	VP7	ClaI-HhaI DraI-EcoRV HinfI-EcoRV EcoRV-HincII	vaccUKtc.VP7(ClaI-HhaI) vaccUKtc.VP7(DraI-EcoRV) vaccUKtc.VP7(HinfI-EcoRV) vaccUKtc.VP7(EcoRV-HincII)	R. Heath F. Xu R. Heath F. Xu
UKtc	NS34		vaccUktc.NS34	S. Stagg
UKtc	NS28		vaccUKtc.NS28	S. Stagg
UKtc	NS26		vaccUKtc.NS26	R. Heath
UKtc	NS12		vaccUKtc.NS12	R. Heath

fragments of rotavirus UKtc gene 8 (VP7 protein), ClaI-HhaI and HinfI-EcoRV were constructed.

Table 9 shows all the recombinant vaccinia viruses which were used in this project and the name of the constructor. All the recombinants were plaque purified before growing to high titre stock and were used to infect target cells in ^{51}Cr-release assays to measure CTL activity.

CHAPTER 7

ANALYSIS AND EXPRESSION OF RECOMBINANT VACCINIA VIRUSES

7.1 AIMS

The aim of the work described in this chapter was first to confirm the nucleotide sequence of the fragments of rotavirus UKtc gene 8 (VP7) within the recombinant vaccinia virus genome. Of particular importance was the sequence at the vector-insert 5' junction within the recombinant vaccinia virus which allows in-frame translation of the insert using the vector ATG. The second aim was to confirm the expression of the rotavirus protein in cells infected with recombinant vaccinia virus. Immunofluorescence was used to detect the expression of vaccUKtc.VP2, VP3, NS26 and NS12.

7.2 SEQUENCE ANALYSIS OF RECOMBINANT VACCINIA VIRUSES CONTAINING FRAGMENTS OF UKTC GENE 8 (VP7)

VaccUKtc.VP7(ClaI-HhaI), (DraI-EcoRV), (HinfI-EcoRV) and (EcoRV-HincII) were used to infect separate wells of a 6-well tissue culture dish of confluent CV-1 cells at an moi of ≤ 0.1. After two days of incubation, viral DNA was extracted and PCR amplification performed using the 5' $P_{7.5}$ and 3' TK primers (see Table 5). The amplified PCR products were excised from the agarose gel and purified using the Gene Clean® II purification kit. The DNA products were sequenced by L. Ward, University of Warwick, using an automated DNA sequencer. Two separate sequencing reactions were performed on the DNA, using either the 5' $P_{7.5}$ or 3' TK primer, to span the fragment.

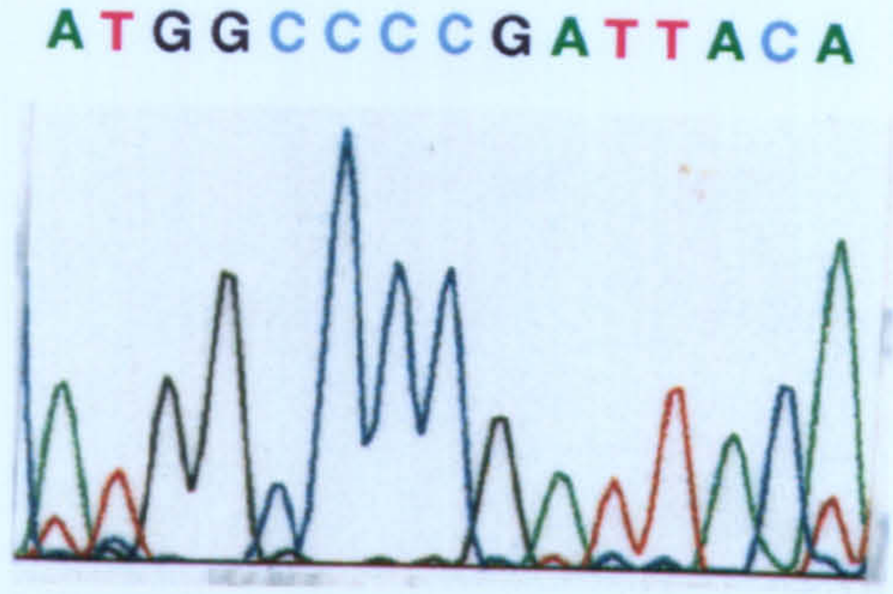
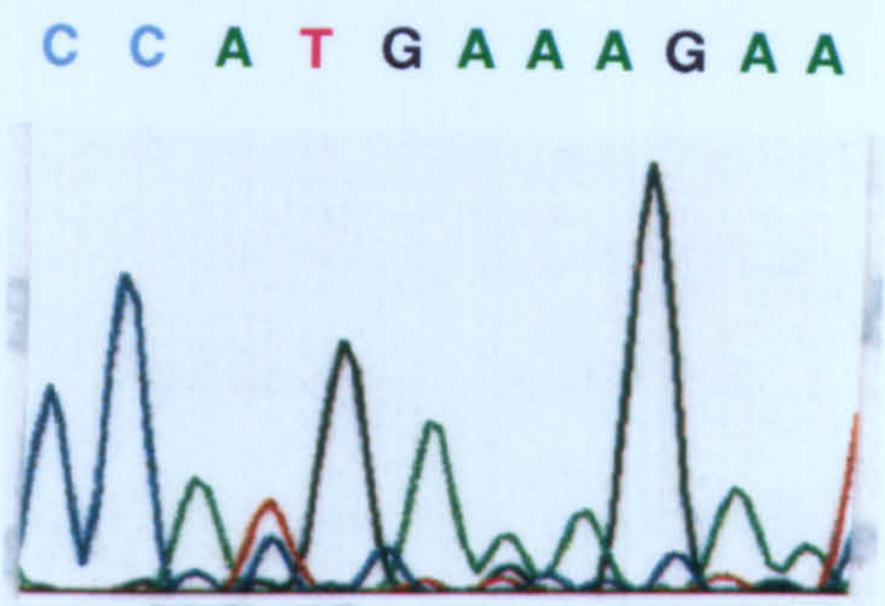
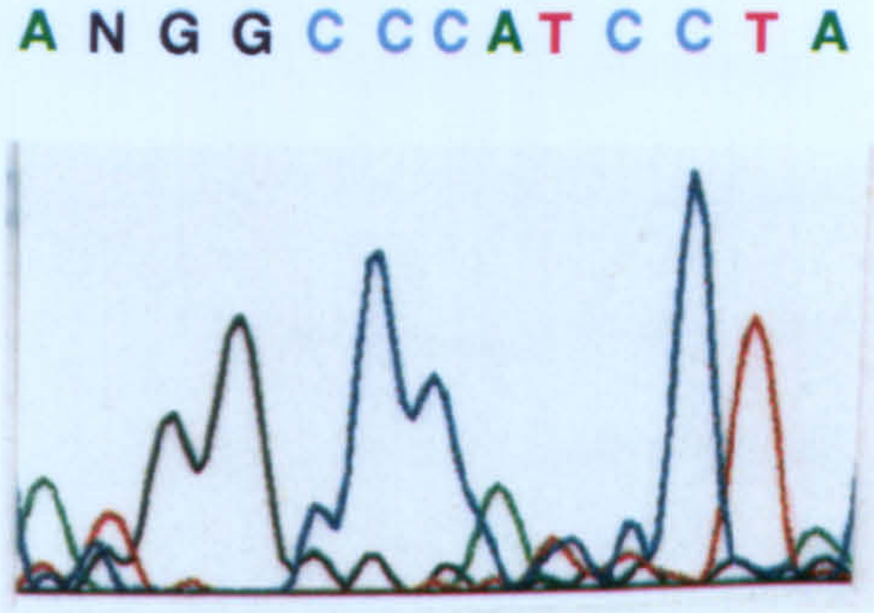
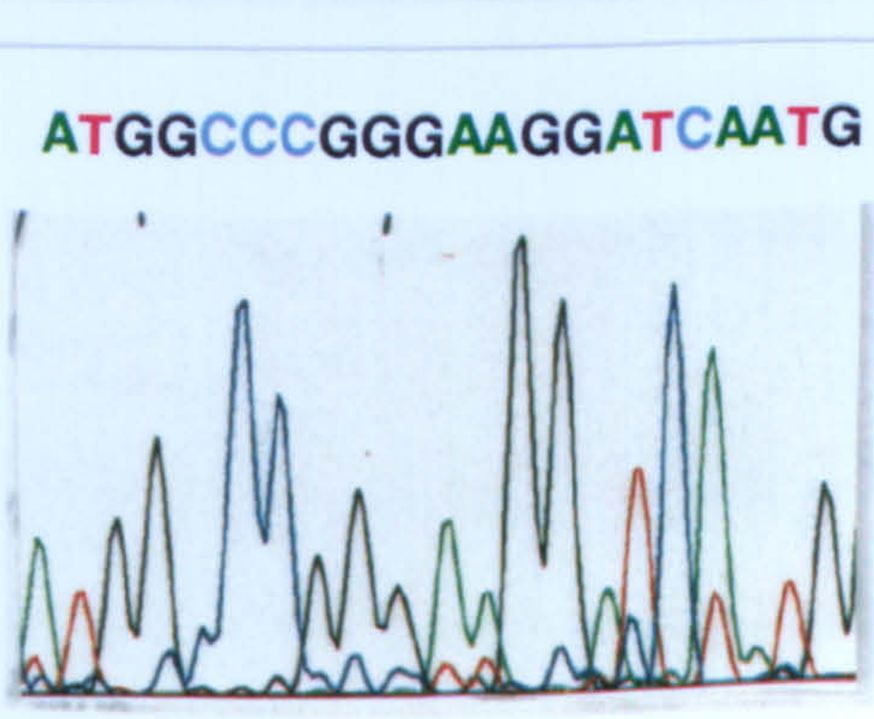
Figure 23 shows the predicted vector-insert (5' end) sequence within the vaccinia virus genome and the corresponding data from the automated sequencer. The results indicate that the 5' end of all four gene 8 fragments contained the predicted junction required for in-frame translation from the initiating ATG (provided by the vector). The sequence data spanning the fragments from both ends also confirmed that, although base changes were observed (due to bona-fide gene mutations or sequencing errors), no stop codons had been introduced into the gene 8 fragments (data not shown).

FIGURE 23. SEQUENCE ANALYSIS OF VECTOR-INSERT 5' JUNCTION WITHIN RECOMBINANT VACCINIA VIRUS TK⁻ GENE

Recombinant vaccinia viruses containing fragments of rotavirus UKtc gene 8, vaccUKtc.VP7(ClaI-HhaI), (DraI-EcoRV), (HinfI-EcoRV) and (EcoRV-HincII) were used to inoculate cells at an moi of ≤ 0.1. After two days of incubation the DNA samples were extracted as described in Materials and Methods. 5' $P_{7.5}$ and 3' TK primers (see Table 5) were used to PCR amplify the gene 8 fragment from each DNA sample. Following agarose gel electrophoresis, the PCR product was excised from the gel and purified as described in Materials and Methods. The PCR product was sequenced using the 5' $P_{7.5}$ primer and the 3' TK primer in separate reactions. Automated sequencing was carried out as described in section 4.3.12 of materials and methods by L. Ward, University of Warwick.

The first column shows the names of the recombinant vaccinia viruses with their predicted vector-insert junction for in-frame translation from the vector ATG. The second column shows the results obtained from the automated sequencer.

FIGURE 23

RECOMBINANT NAME WITH PREDICTED SEQUENCE	AUTOMATED SEQUENCER DATA
VaccUKtc.VP7(ClaI-HhaI) ATG GCC C ⋮ CG ATT ACA	
VaccUKtc.VP7(DraI-EcoRV) CC ATG ⋮ AAA GAA	
VaccUKtc.VP7(HinfI-EcoRV) ATG GCC C ⋮ AT TCT A	
VaccUKtc.VP7(EcoRV-HincII) ATG GCC CGG GAA GG ⋮ A TCA ATG	

7.3 ROTAVIRUS PROTEIN EXPRESSION FOLLOWING RECOMBINANT VACCINIA VIRUS INFECTION OF CELLS

The expression of the complete set of rotavirus UKtc proteins was checked using immunofluorescence by Prof. M. McCrae and N. Broughton, University of Warwick. Expression of the VP2, VP3, NS26 and NS12 proteins was of particular importance because the corresponding recombinant vaccinia viruses were constructed in the current project (see Table 9).

Confluent BS-C-1 cells were grown on glass coverslips in a 12-well tissue culture dish. Individual wells were infected with either (i) WT vaccinia virus at an moi of 0.002 (negative control), (ii) UKtc rotavirus at an moi of 0.1 (positive control), (iii) vaccUKtc.VP7 at an moi of 0.002 (positive control) or (iv) vaccUKtc.VP2, VP3, VP6, NS26 and NS12 (one recombinant per well) at an moi of 0.002. Cells were fixed after 7 hours incubation and rabbit anti-rotavirus primary antibody was then added. After washing, biotinylated anti-rabbit secondary antibody was added to the cells, followed by streptavidin fluorescein. The cells were washed again and viewed under a UV microscope.

Figure 24 shows photographs taken of the virus controls and the recombinant vaccinia viruses under examination. The results show no fluorescence when cells were infected with the WT vaccinia virus (negative control) and bright fluorescence with the UKtc rotavirus (positive control) (Figure 24A and B). The vaccUKtc.VP7 was also used as a positive control since there was a VP7-specific CTL response against target cells infected with this recombinant (see Chapter 8) and, indeed, fluorescence was seen in the vaccUKtc.VP7 infected cells (Figure 24C).

VaccUKtc.VP2, VP3, NS26 and NS12 infected cells, although exhibiting a relatively weak fluorescence signal, were deemed positive for rotavirus protein expression (Figure 24D, E, G and H) and, indeed, expression was confirmed by a VP3-specific CTL

FIGURE 24. IMMUNOFLUORESCENCE OF ROTAVIRUS PROTEINS (VP2, VP3, VP6, NS26 AND NS12) EXPRESSED IN RECOMBINANT VACCINIA VIRUS INFECTED CELLS

Confluent BS-C-1 cells were grown on glass coverslips in a 12-well tissue culture dish. Individual wells were infected with either (i) WT vaccinia virus at an moi of 0.002 (positive control), (ii) UKtc rotavirus at an moi of 0.1 (positive control), (iii) vaccUKtc.VP7 at an moi of 0.002 (positive control) or (iv) vaccUKtc.VP2, VP3, VP6, NS26 and NS12 (one recombinant per well) at an moi of 0.002. Immunofluorescence was performed as described in section 4.4.4 of Materials and Methods by Prof. M. McCrae and N. Broughton, University of Warwick. All cells were photographed using a UV microscope at X20 objective magnification.

Panel A: Wild-type vaccinia virus (WR^+) (negative control)

Panel B: UKtc rotavirus (positive control)

Panel C: vaccUKtc.VP7 (positive recombinant control)

Panel D: vaccUKtc.VP2

Panel E: vaccUKtc.VP3

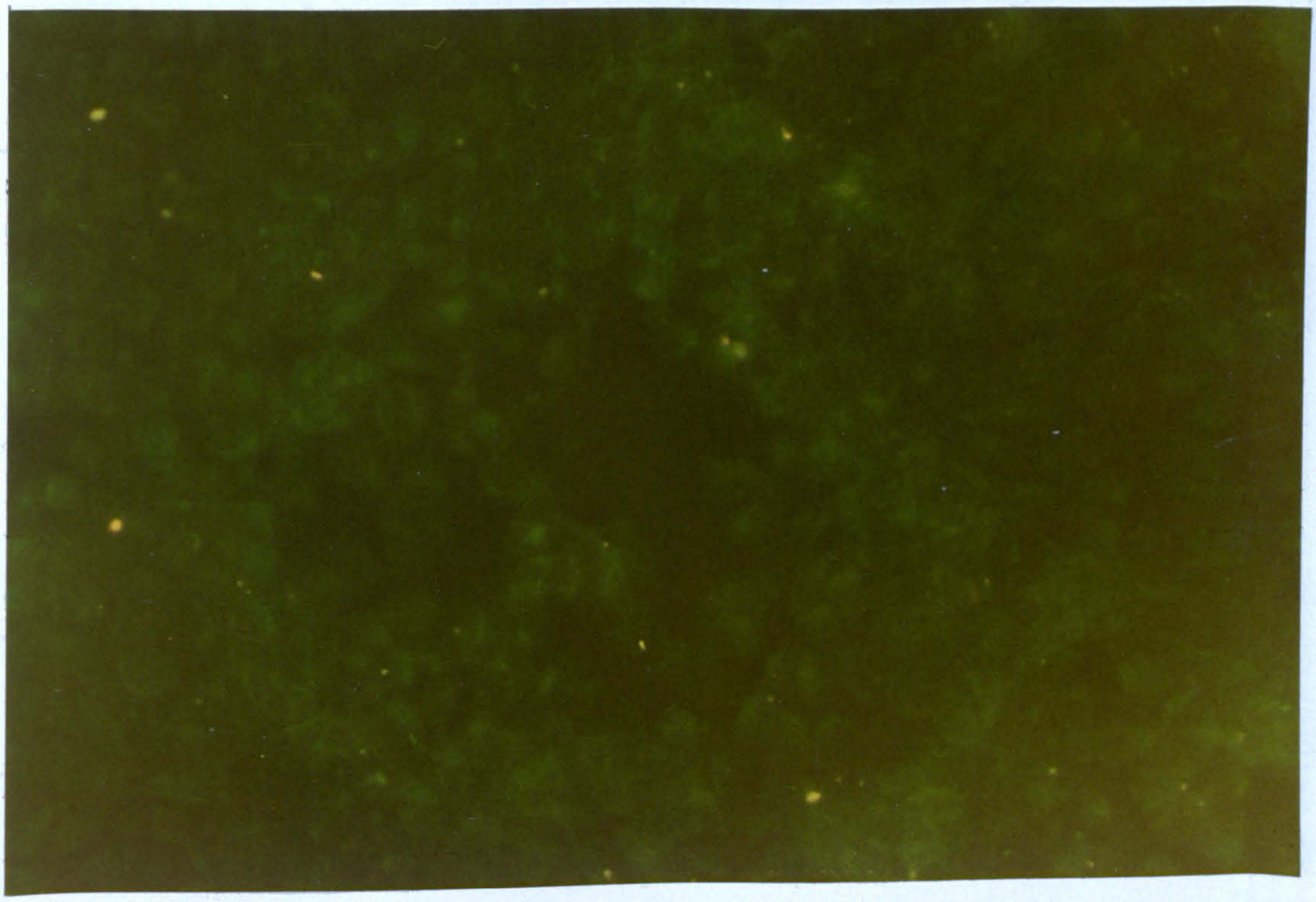
Panel F: vaccUKtc.VP6

Panel G: vaccUKtc.NS26

Panel H: vaccUKtc.NS12

FIGURE 24

(A)



(B)

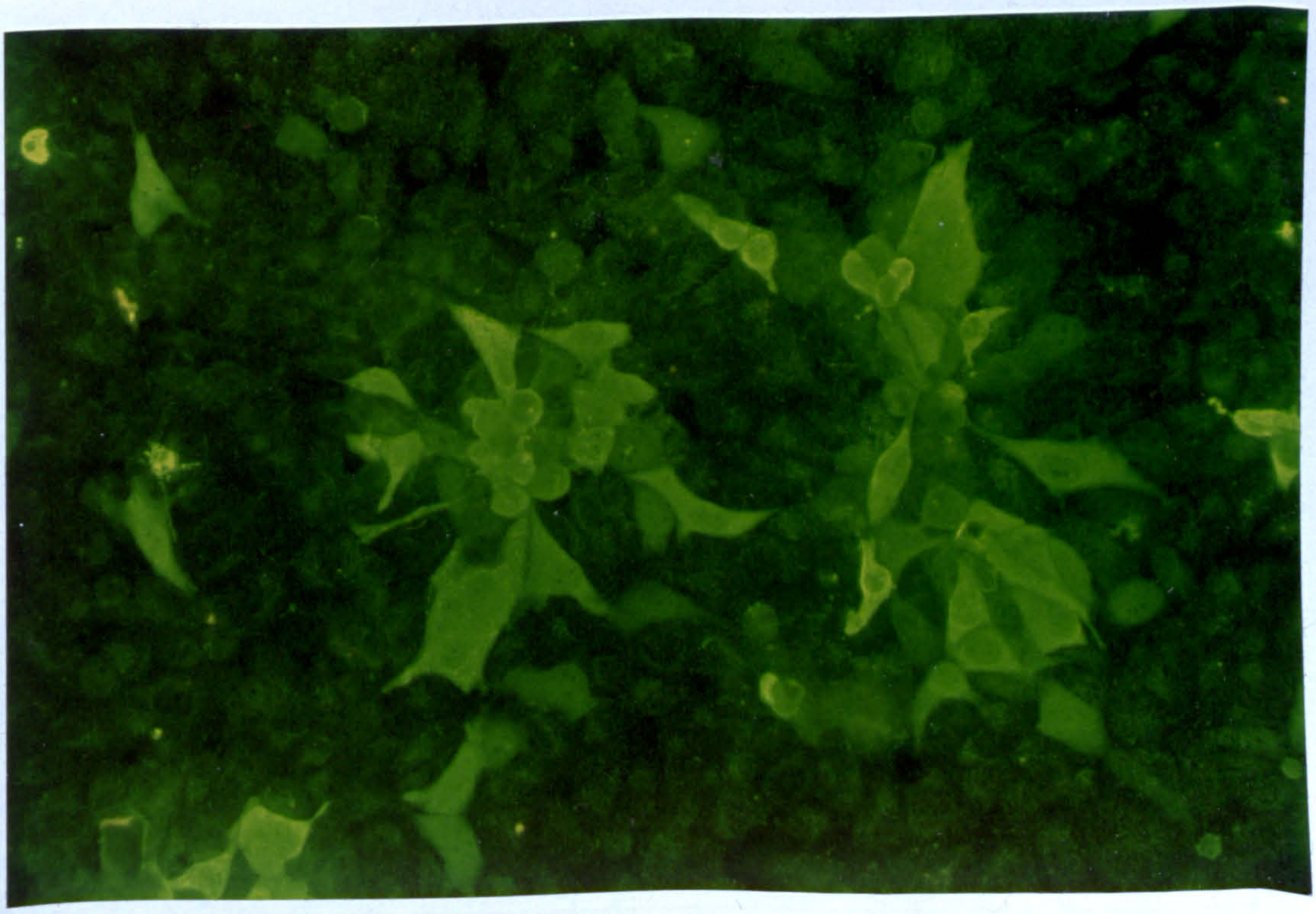
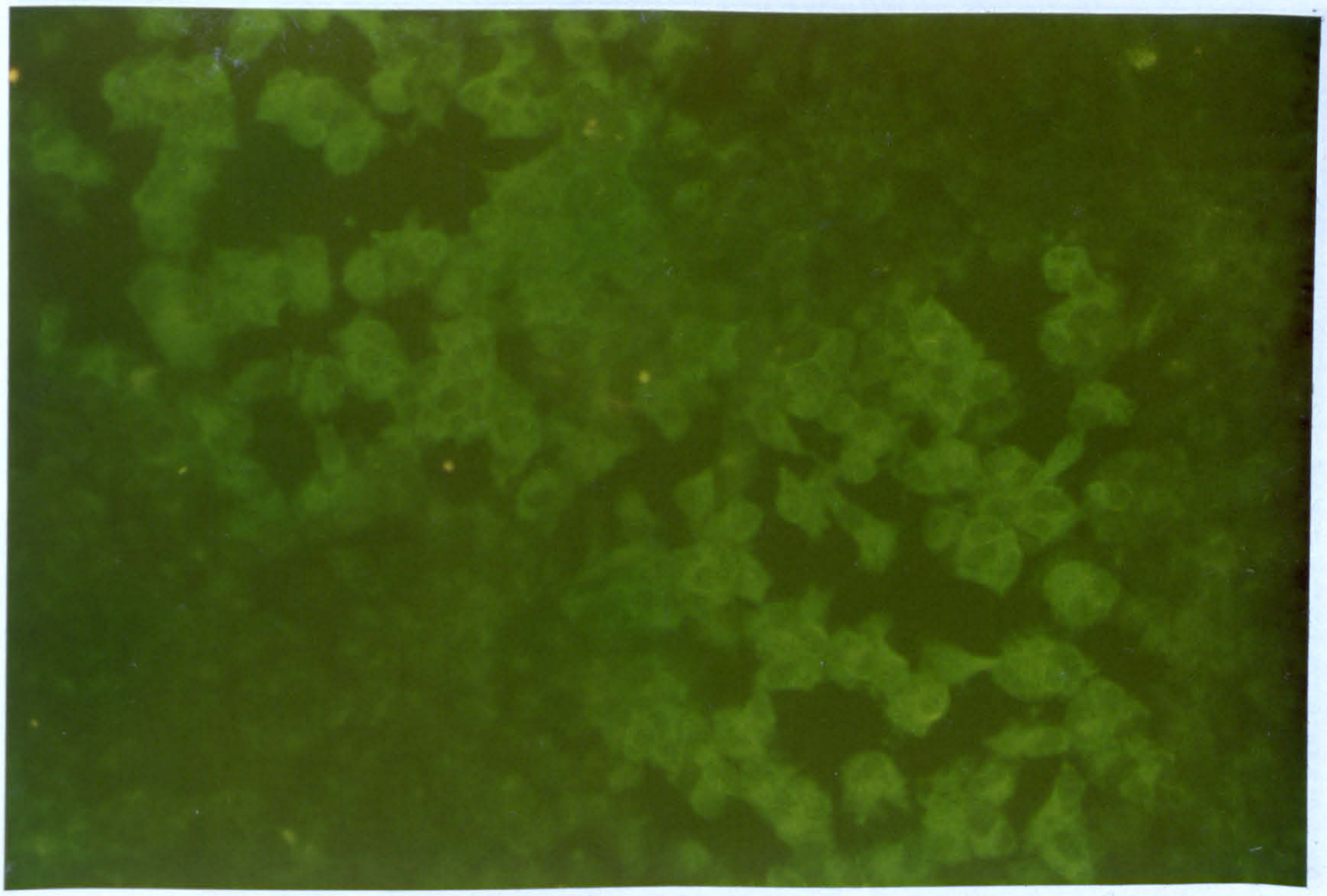


FIGURE 24. (CONTINUED)

(C)



(D)

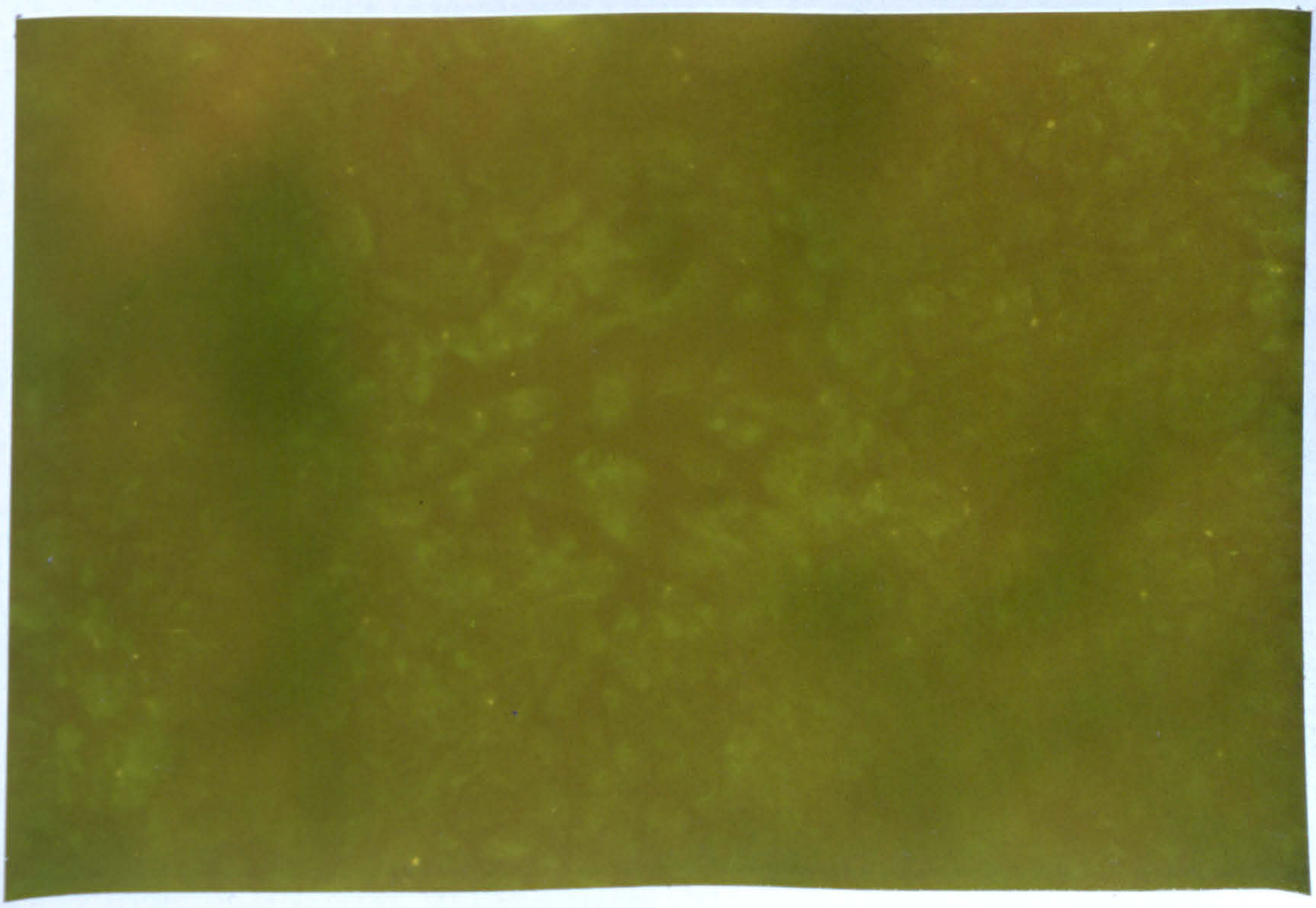
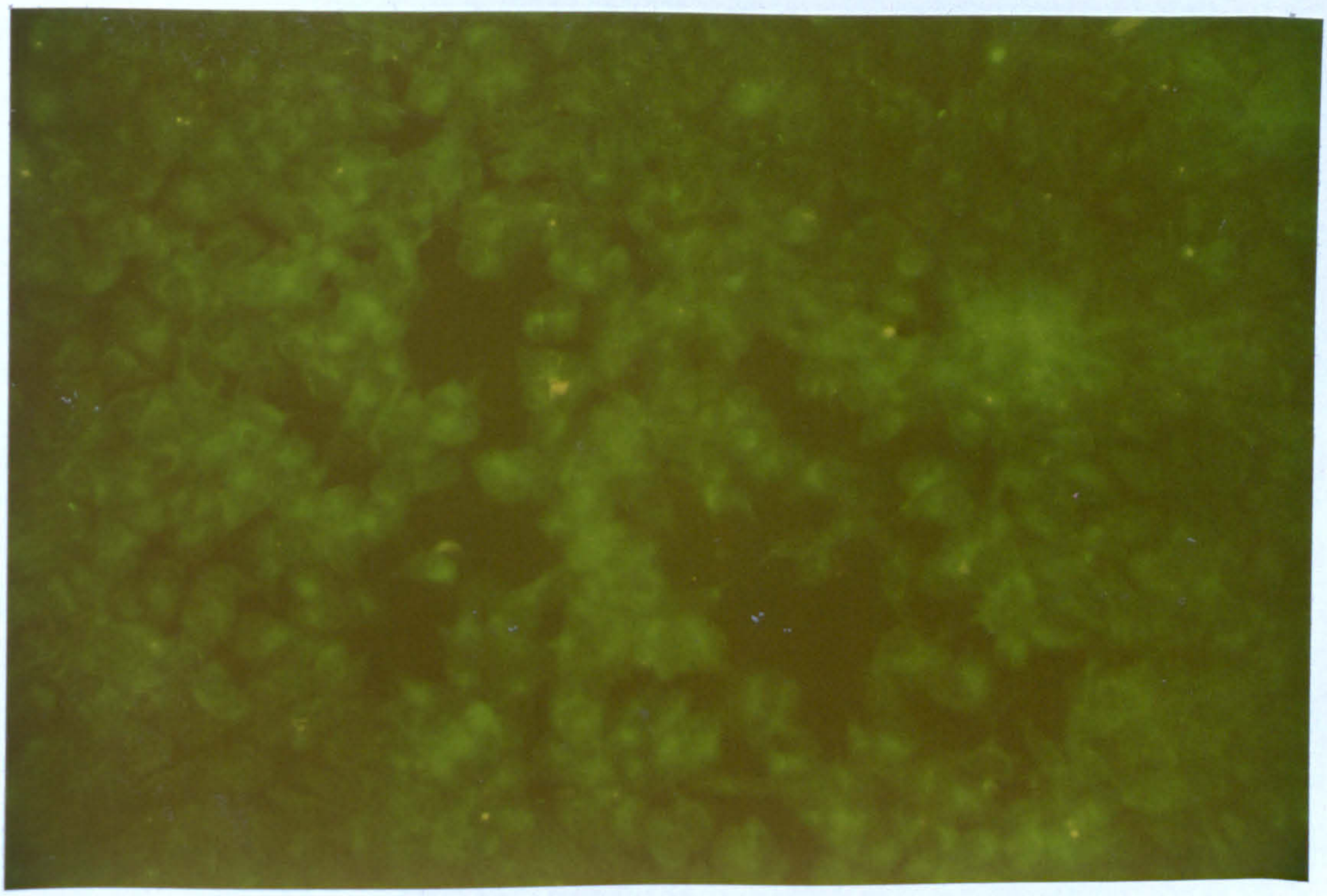


FIGURE 24. (CONTINUED)

(E)



(F)

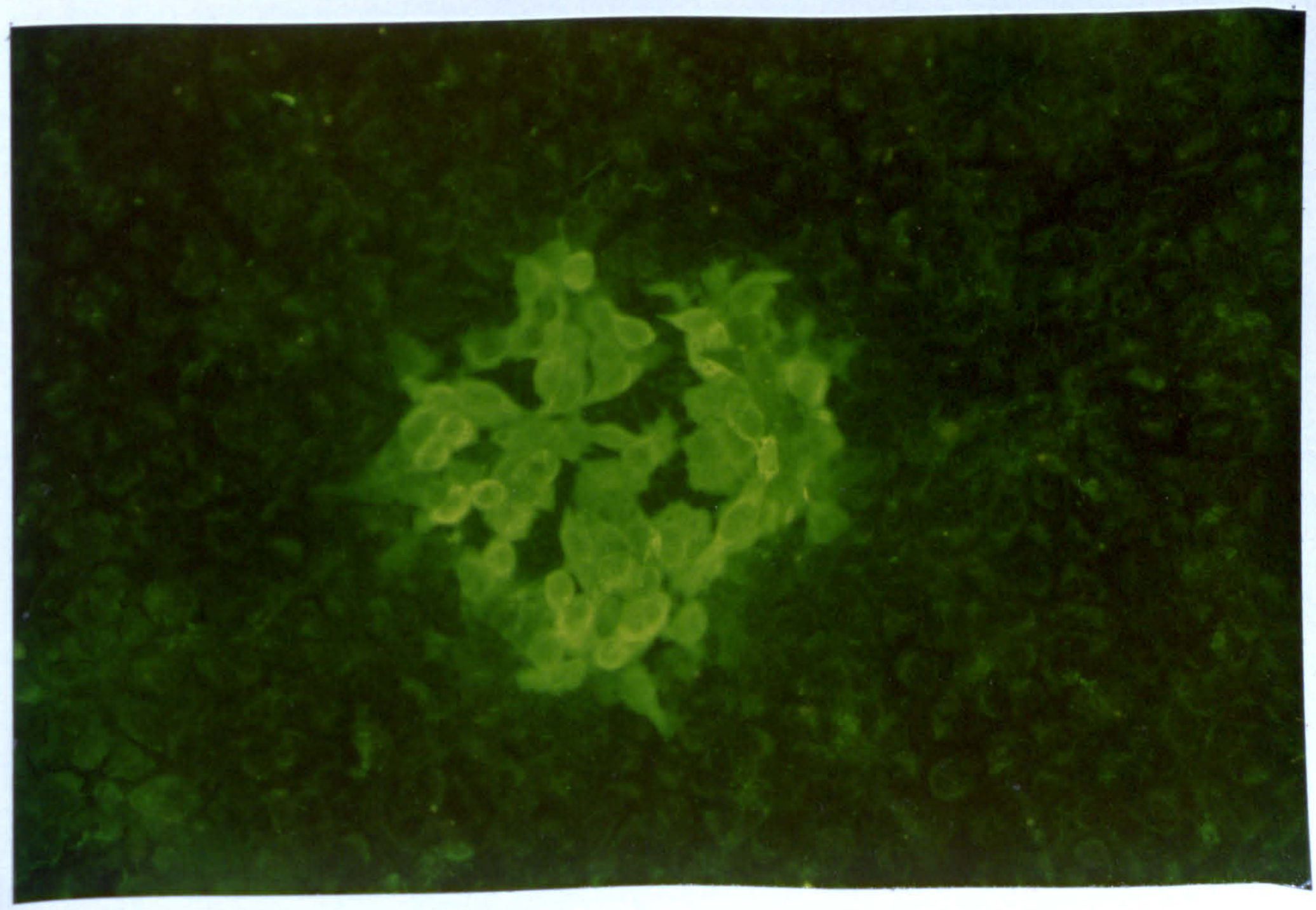
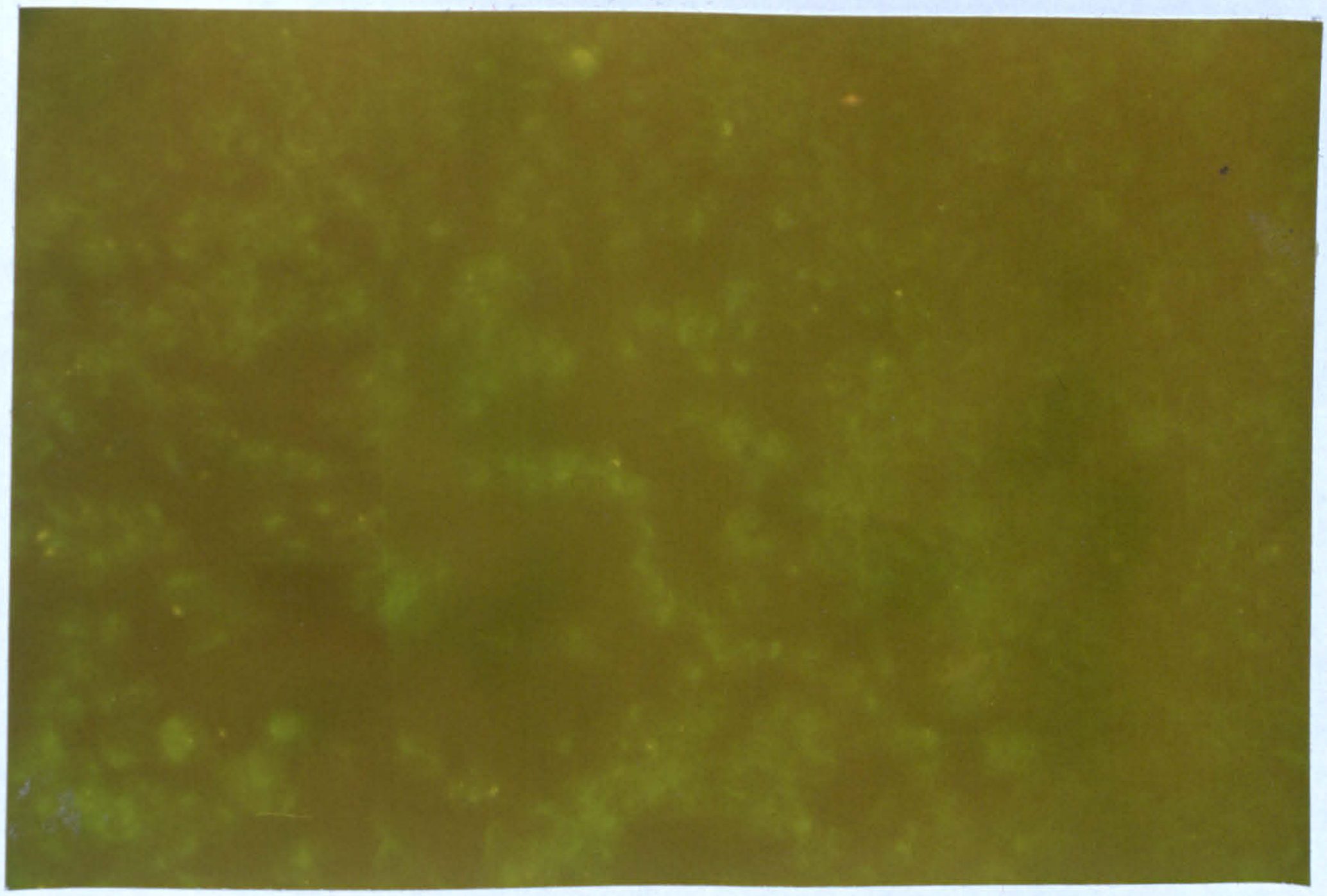
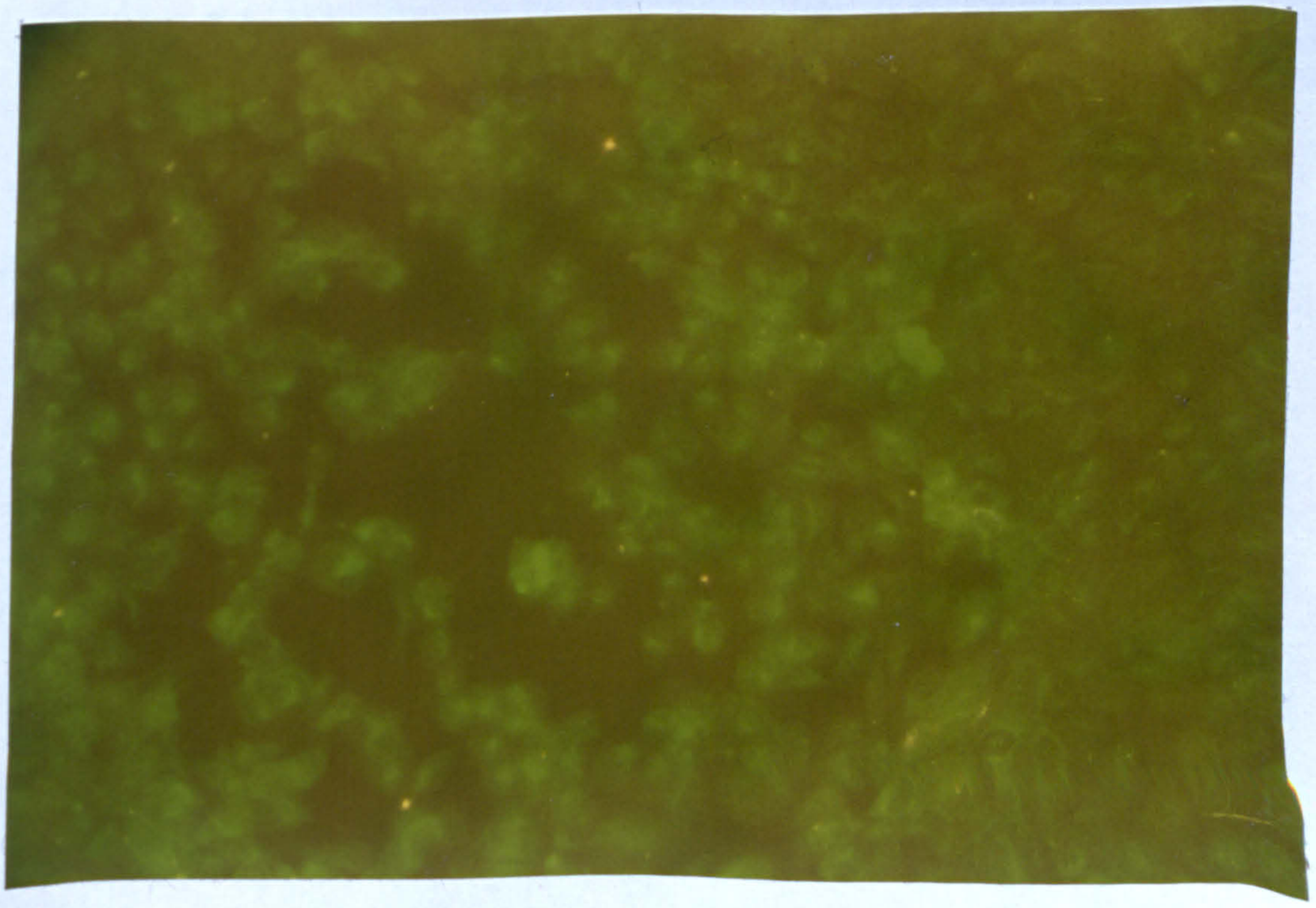


FIGURE 24. (CONTINUED)

(G)



(H)



response against target cells infected with vaccUKtc.VP3 (see Chapter 8). This mirrors the experiments by Townsend *et al.* (1984) in which immunoprecipitation of influenza A virus nucleoprotein was only achieved with two out of three transfected L-cell clones despite the nucleoprotein-specific CTL lysis of all three clones. Both these results suggest that the amount of expressed protein required for recognition by CTLs is less than that required for detection by immunofluorescence (or immunoprecipitation). It should be noted that vaccUKtc.VP6 infected cells show very bright fluorescence and were deemed positive for VP6 expression (Figure 24F). In this case, however, there does not appear to be a VP6-specific CTL response against target cells infected with vaccUKtc.VP6 (see Chapter 8).

Immunofluorescence was also used to detect the expression of rotavirus proteins by cells infected with vaccUKtc.VP1, VP4, NS53, NS35, NS34 and NS28. In those experiments NS53 and NS28 fluorescence was strongly positive, whereas, VP1, VP4, NS35 and NS34 gave much weaker fluorescence (data not shown).

7.4 CONCLUSIONS

Sequence analysis has revealed that the recombinant vaccinia viruses containing fragments of UKtc gene 8 have been successfully constructed without the addition/deletion of nucleotides at the vector-insert (5' end) junction within the vaccinia virus genome. The sequence analysis of the gene 8 fragments also showed that no stop codons had been introduced. The sequence data therefore predicts expression of the four rotavirus UKtc VP7 protein fragments in target cells infected with the corresponding recombinant vaccinia viruses.

Immunofluorescence of rotavirus proteins in cells infected with vaccUKtc.VP2, VP3, NS26 and NS12 was weakly positive. These recombinant vaccinia viruses can, therefore, be used in the ^{51}Cr-release assay to measure specific CTL activity to individual rotavirus proteins.

CHAPTER 8

THE CTL RESPONSE TO INDIVIDUAL ROTAVIRUS UKtc PROTEINS IN THREE MHC CLASS I HAPLOTYPES

8.1 AIMS

The initial task of this project was aimed at extending work carried out in the group on the CTL response to rotavirus (S. Stagg, personal communication) and to complete the coverage of the rotavirus UKtc proteins not dealt with in that work, i.e., VP2, VP3, NS26 and NS12. In addition, previous studies on the CTL response to rotavirus have been confined to single class I mouse haplotypes (Franco *et al.*, 1994; Offit *et al.*, 1994) and, therefore, a primary objective of this project was to gauge the likely applicability of those observations for an outbred population in which any CTL-based vaccine would be used.

8.2 RATIONALE TO THE ^{51}CR-RELEASE ASSAY

The CTL response is routinely measured in viral systems using the ^{51}Cr-release assay. This assay utilises the fact that the ^{51}Cr-isotope (sodium chromate, $Na_2{}^{51}CrO_4$) is taken up by cells in the hexavalent form and released from lysed cells in the trivalent form, which is not reutilised.

The ^{51}Cr-release assay utilises two cell populations, effectors and targets (see Figure 25). The effectors are generated from splenocytes taken from *in vivo* inoculated animals (responder cells) which are *in vitro* stimulated with naive splenocytes presenting the inoculating virus (stimulator cells). The targets (which, in principle, may be any MHC class I matched cell) are loaded with ^{51}Cr and normally infected with the inoculating virus. The effector cells recognise processed antigen presented on the surface of the target cells in association with MHC class I and this leads to cell binding and subsequent target cell lysis. The released ^{51}Cr is measured and expressed as the percentage specific lysis.

FIGURE 25. THE ^{51}CR-RELEASE ASSAY FOR CTL ACTIVITY

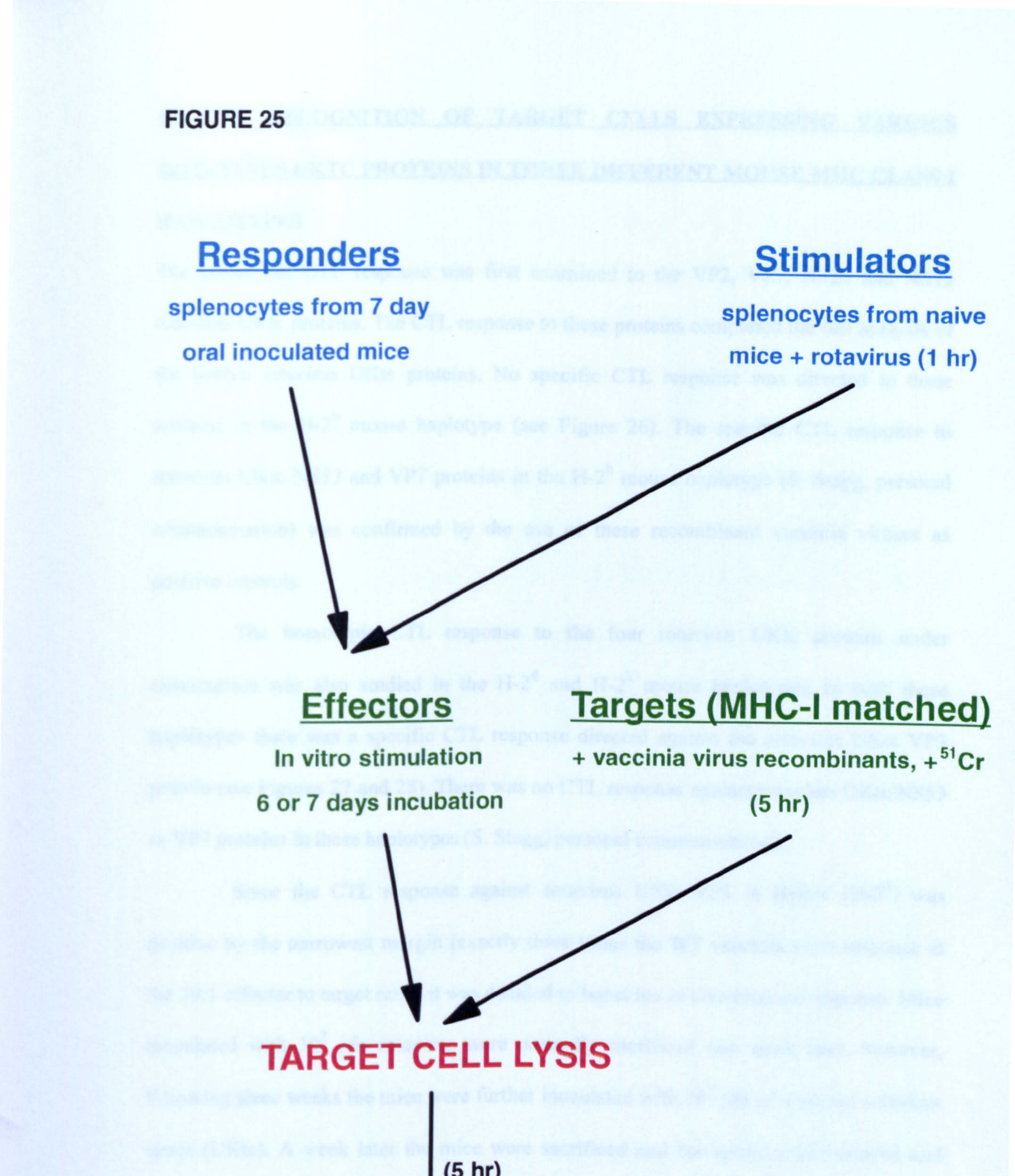
Two 6 to 10 week old female mice were orally inoculated with 10^7 pfu of rotavirus and sacrificed 7 days later. The spleens were removed and splenocytes prepared at 6 X 10^6 cells/ml (responders). 2 X 10^7 splenocytes from two naive 6 to 10 week old female mice were infected with the inoculating rotavirus strain at an moi of 1 for 1 hr (stimulators). 6 X 10^6 responder cells were cultured *in vitro* with stimulator cells at a ratio of 2:1 for 6 or 7 days (effectors) as described in sections 4.6.1 to 4.6.3 of Materials and Methods.

1 X 10^6 MHC class I matched cells were loaded with 50 μCi ^{51}Cr and infected with either wild-type (WT) vaccinia virus strain WR^+ or recombinant vaccinia virus at an moi of 10 for 5 hr (targets), as described in section 4.6.4 of Materials and Methods.

1 X 10^4 target cells were mixed with (i) effector cells at ratios of 1:1, 3:1, 10:1 and 30:1 (effector:target) (test ^{51}Cr-release), (ii) medium (spontaneous ^{51}Cr-release) or (iii) detergent (total ^{51}Cr-release), in 200 μl volume for 5 hr. 100 μl of supernatant was removed and the ^{51}Cr-release measured on a γ-radiation counter as described in section 4.6.5 of Materials and Methods.

The % specific lysis was calculated for each effector:target ratio, as described in section 4.6.6 of Materials and Methods, and the results displayed on a bar graph. Positive CTL killing of target cells (infected with recombinant vaccinia virus) was defined by (i) a minimum 10% specific lysis, and (ii) a minimum threefold greater % specific lysis than the negative control (target cells infected with WT vaccinia virus). To confirm any positive results, all assays were repeated at least twice.

FIGURE 25


Responders
splenocytes from 7 day
oral inoculated mice
Stimulators
splenocytes from naive
mice + rotavirus (1 hr)
Effectors
In vitro stimulation
6 or 7 days incubation
Targets (MHC-I matched)
+ vaccinia virus recombinants, + 51Cr
(5 hr)
TARGET CELL LYSIS
(5 hr)
Measure 51Cr release
on γ-radiation counter

8.3 CTL RECOGNITION OF TARGET CELLS EXPRESSING VARIOUS ROTAVIRUS UKTC PROTEINS IN THREE DIFFERENT MOUSE MHC CLASS I HAPLOTYPES

The homotypic CTL response was first examined to the VP2, VP3, NS26 and NS12 rotavirus UKtc proteins. The CTL response to these proteins completed the full analysis of the twelve rotavirus UKtc proteins. No specific CTL response was directed to these proteins in the H-2^b mouse haplotype (see Figure 26). The specific CTL response to rotavirus UKtc NS53 and VP7 proteins in the H-2^b mouse haplotype (S. Stagg, personal communication) was confirmed by the use of these recombinant vaccinia viruses as positive controls.

The homotypic CTL response to the four rotavirus UKtc proteins under examination was also studied in the H-2^d and H-2^k mouse haplotypes. In both these haplotypes there was a specific CTL response directed against the rotavirus UKtc VP3 protein (see Figures 27 and 28). There was no CTL response against rotavirus UKtc NS53 or VP7 proteins in these haplotypes (S. Stagg, personal communication).

Since the CTL response against rotavirus UKtc VP3 in Balb/c (H-2^d) was positive by the narrowest margin (exactly three times the WT vaccinia virus response at the 30:1 effector to target ratio) it was decided to boost the *in vivo* immune response. Mice inoculated with 10^7 pfu rotavirus were normally sacrificed one week later, however, following three weeks the mice were further inoculated with 10^7 pfu of a second rotavirus strain (UKtc). A week later the mice were sacrificed and the splenocytes removed and stimulated *in vitro* as normal. It was found that this inoculation schedule gave an enhanced indication of the CTL response to rotavirus UKtc VP3 in Balb/c mice (twelve times the WT vaccinia virus response at the 30:1 effector to target ratio) (see Figure 29). Because of the 'quantal' nature of the CTL response (CTLs being either lytic or not) it is believed that repeated immunisation increases the lytic and recycling rates of CTLs. For example,

FIGURE 26. % SPECIFIC LYSIS OF TARGETS EXPRESSING VARIOUS UKTC PROTEINS BY UKTC PRIMED C57BL/6 (H-2^b) EFFECTORS

Two 6 to 10 week old female C57BL/6 mice (H-2^b) were orally inoculated with 10^7 pfu rotavirus UKtc (serotype 6) and 7 days later were sacrificed and the spleens removed. The splenocytes were *in vitro* stimulated to produce the effector cells as described in Figure 25.

EL4 target cells (H-2^b) were loaded with ^{51}Cr, infected with WT vaccinia virus or recombinant vaccinia viruses [vaccUKtc.VP2, VP3, NS53 (positive control), VP7 (positive control), NS26 or NS12] and the ^{51}Cr-release assays set up as described in Figure 25.

FIGURE 27. % SPECIFIC LYSIS OF TARGETS EXPRESSING VARIOUS UKTC PROTEINS BY UKTC PRIMED BALB/C (H-2^d) EFFECTORS

Two 6 to 10 week old female Balb/c mice (H-2^d) were orally inoculated with 10^7 pfu rotavirus UKtc (serotype 6) and 7 days later were sacrificed and the spleens removed. The splenocytes were *in vitro* stimulated to produce the effector cells as described in Figure 25.

P815 target cells (H-2^d) were loaded with ^{51}Cr, infected with WT vaccinia virus or recombinant vaccinia viruses (vaccUKtc.VP2, VP3, NS26 or NS12) and the ^{51}Cr-release assays set up as described in Figure 25.

FIGURE 26

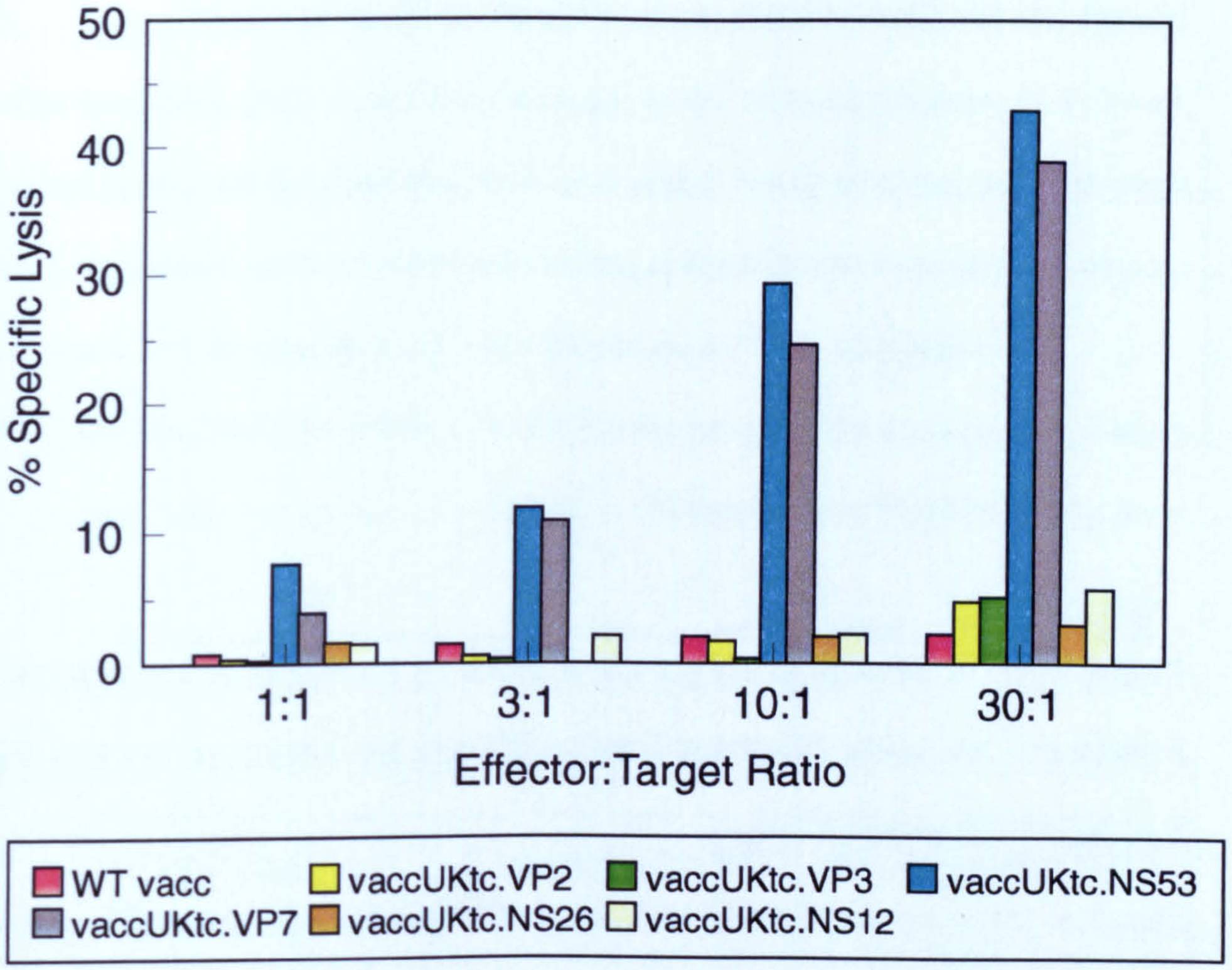


FIGURE 27

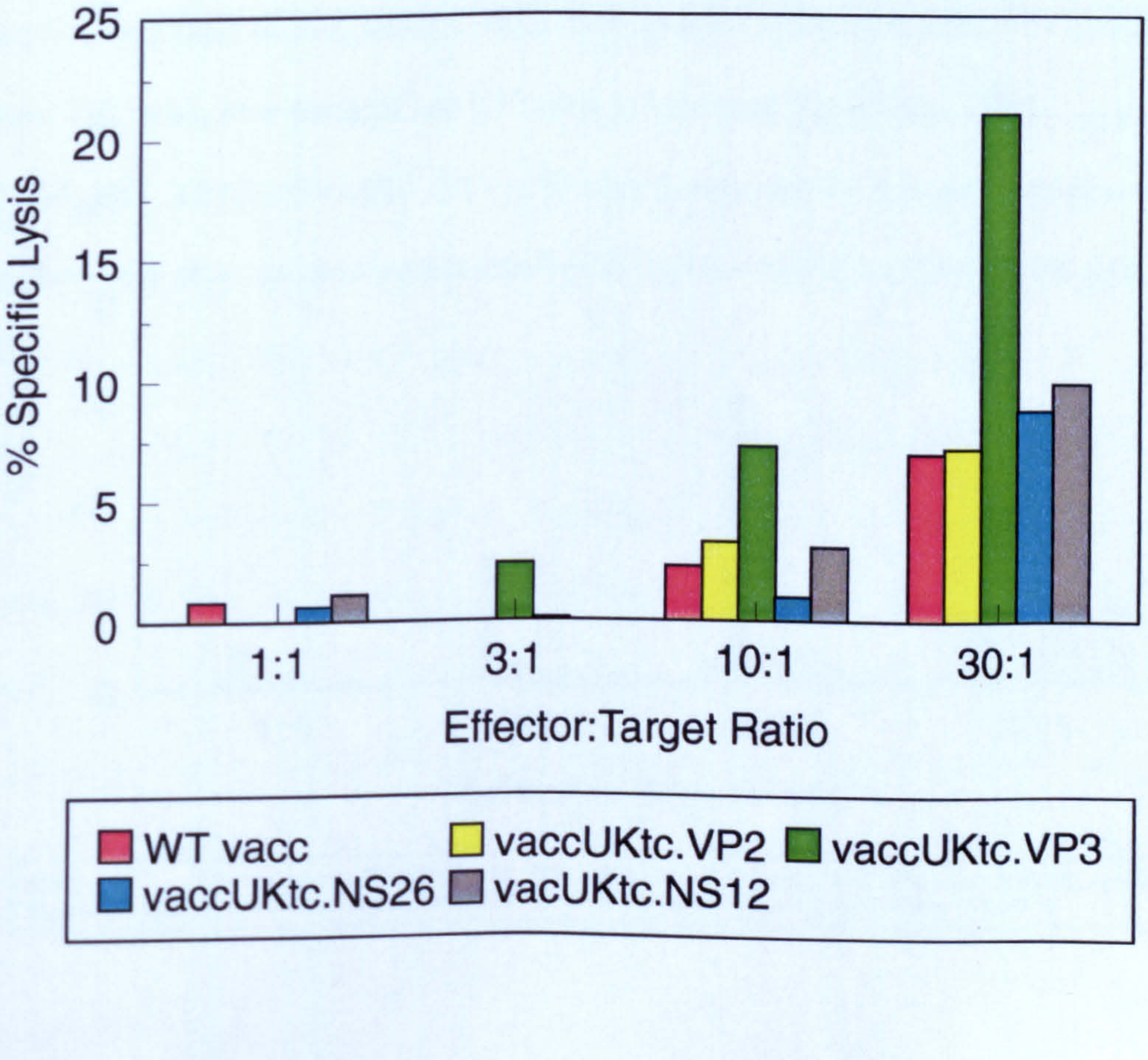


FIGURE 28. % SPECIFIC LYSIS OF TARGETS EXPRESSING VARIOUS UKTC PROTEINS BY UKTC PRIMED C3H/HE,MG (H-2^k) EFFECTORS

Two 6 to 10 week old female C3H/He,mg mice (H-2^k) were orally inoculated with 10^7 pfu rotavirus UKtc (serotype 6) and 7 days later were sacrificed and the spleens removed. The splenocytes were *in vitro* stimulated to produce the effector cells as described in Figure 25.

Ltk$^-$ target cells (H-2^k) were loaded with ^{51}Cr, infected with WT vaccinia virus or recombinant vaccinia viruses (vaccUKtc.VP2, VP3, NS26 or NS12) and the ^{51}Cr-release assays set up as described in Figure 25.

FIGURE 29. % SPECIFIC LYSIS OF TARGETS EXPRESSING VARIOUS UKTC PROTEINS BY RRV (3 WEEKS) FOLLOWED BY UKTC (1 WEEK) PRIMED BALB/C (H-2^d) EFFECTORS

Two 6 to 10 week old female Balb/c mice (H-2^d) were orally inoculated with 10^7 pfu rotavirus RRV (serotype 3) and after 3 weeks orally inoculated with 10^7 pfu rotavirus UKtc (serotype 6). 7 days later the mice were sacrificed and the spleens removed. The spenocytes were *in vitro* stimulated using UKtc to produce the effector cells as described in Figure 25.

P815 cells (H-2^d) were loaded with ^{51}Cr and infected with either WT vaccinia or recombinant vaccinia viruses (vaccUKtc.VP1, VP2, VP3, VP4, NS53, VP6, NS35, VP7, NS34, NS28, NS26 or NS12) and the ^{51}Cr-release assays were set up as described in Figure 25.

FIGURE 28

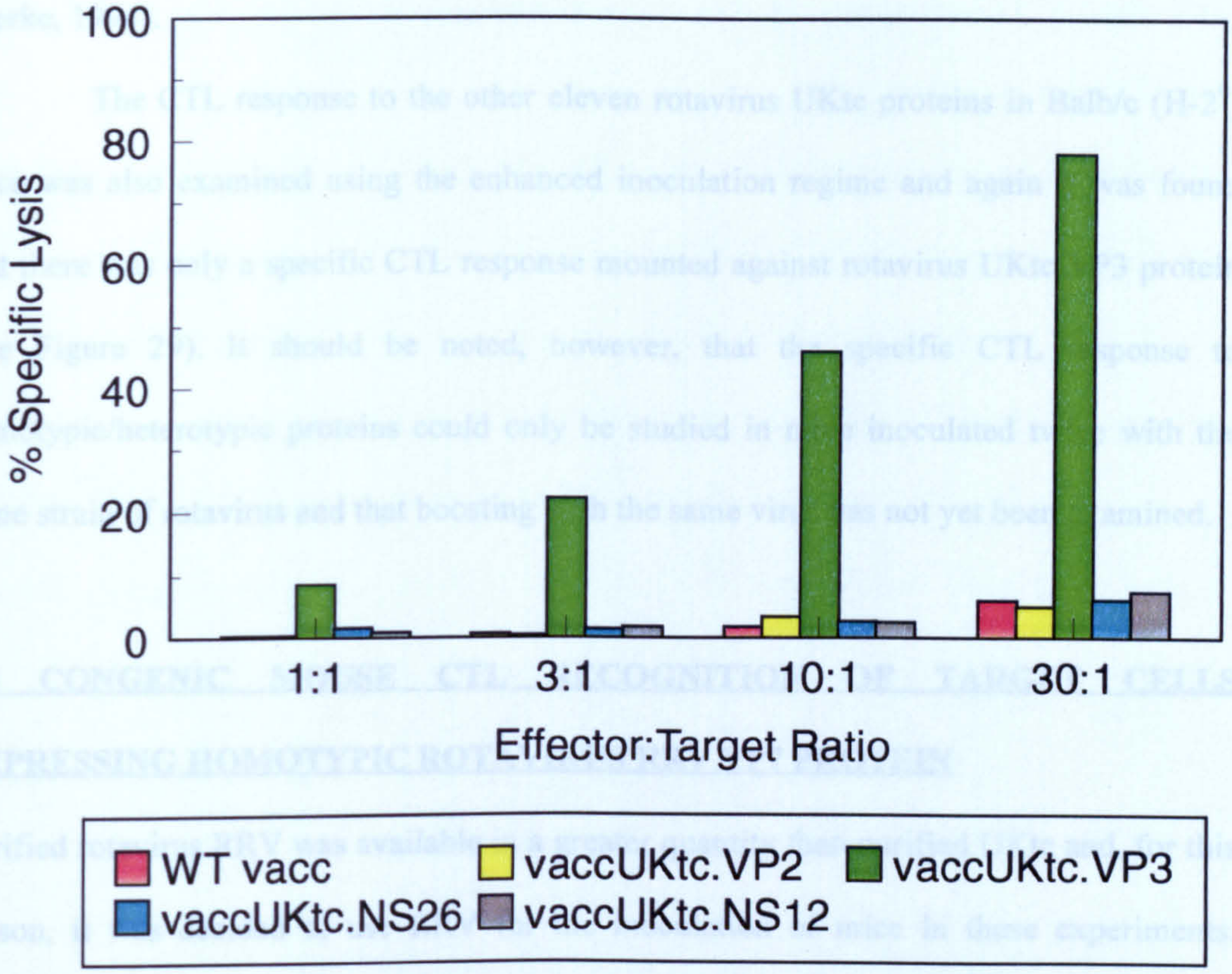
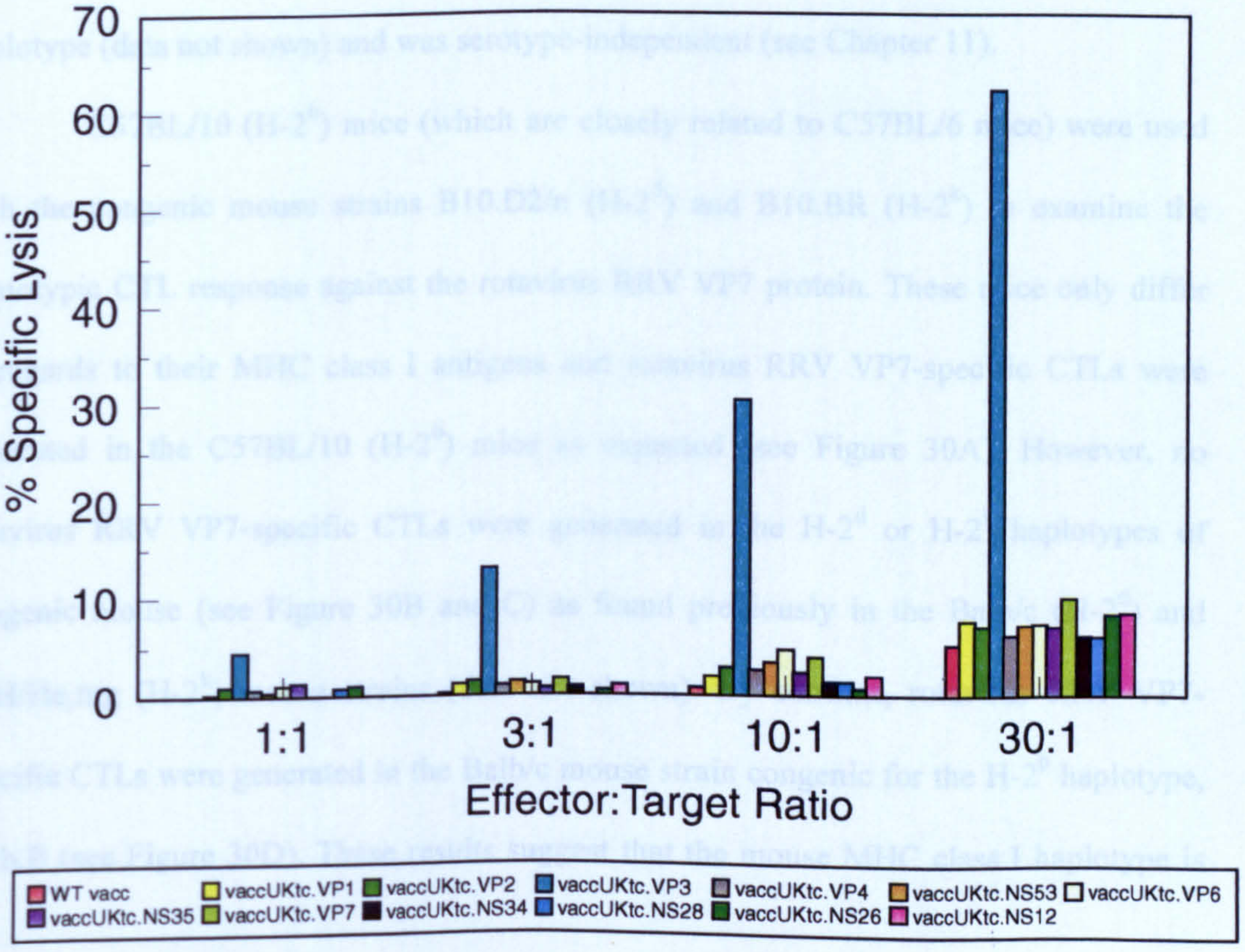


FIGURE 29



hyper-activation of CTL surface molecules may lead to increased target cell binding rates (Berke, 1994).

The CTL response to the other eleven rotavirus UKtc proteins in Balb/c (H-2^d) mice was also examined using the enhanced inoculation regime and again it was found that there was only a specific CTL response mounted against rotavirus UKtc VP3 protein (see Figure 29). It should be noted, however, that the specific CTL response to homotypic/heterotypic proteins could only be studied in mice inoculated twice with the same strain of rotavirus and that boosting with the same virus has not yet been examined.

8.4 CONGENIC MOUSE CTL RECOGNITION OF TARGET CELLS EXPRESSING HOMOTYPIC ROTAVIRUS RRV VP7 PROTEIN

Purified rotavirus RRV was available in a greater quantity than purified UKtc and, for this reason, it was decided to use RRV for the inoculation of mice in these experiments. However, similarly to CTL response to UKtc VP7, it had been established during this study that the RRV primed VP7-specific CTL response was restricted to the H-2^b mouse haplotype (data not shown) and was serotype-independent (see Chapter 11).

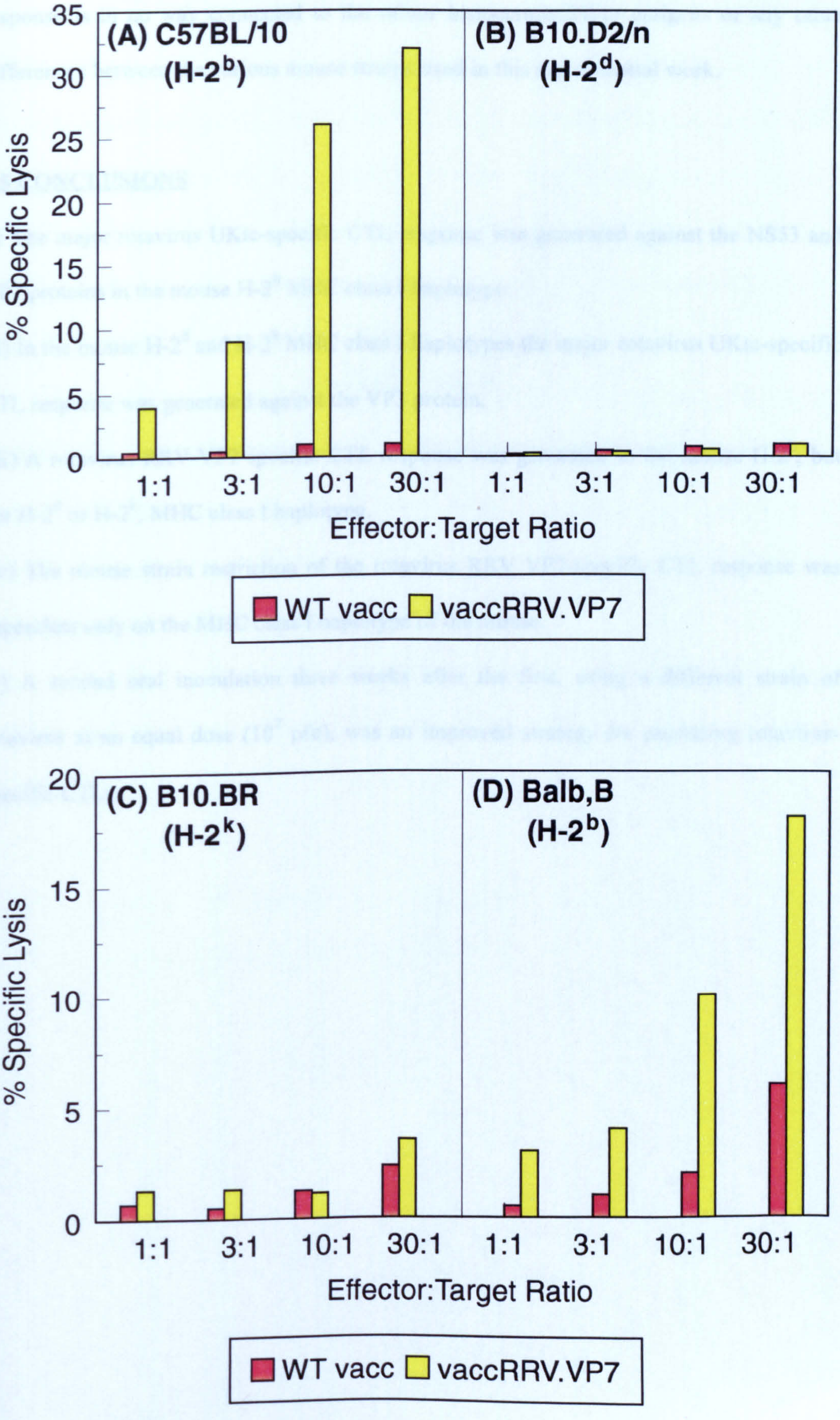
C57BL/10 (H-2^b) mice (which are closely related to C57BL/6 mice) were used with the congenic mouse strains B10.D2/n (H-2^d) and B10.BR (H-2^k) to examine the homotypic CTL response against the rotavirus RRV VP7 protein. These mice only differ in regards to their MHC class I antigens and rotavirus RRV VP7-specific CTLs were generated in the C57BL/10 (H-2^b) mice as expected (see Figure 30A). However, no rotavirus RRV VP7-specific CTLs were generated in the H-2^d or H-2^k haplotypes of congenic mouse (see Figure 30B and C) as found previously in the Balb/c (H-2^d) and C3H/He,mg (H-2^k) mouse strains (data not shown). By contrast, rotavirus RRV VP7-specific CTLs were generated in the Balb/c mouse strain congenic for the H-2^b haplotype, Balb,B (see Figure 30D). These results suggest that the mouse MHC class I haplotype is

FIGURE 30. % SPECIFIC LYSIS OF TARGETS EXPRESSING RRV VP7 PROTEIN BY RRV PRIMED C57BL/10 (H-2^b), B10.D2/N (H-2^d), B10.BR (H-2^k) AND BALB,B (H-2^b) EFFECTORS

Two 6 to 10 week old female mice (A) C57BL/10 (H-2^b), (B) B10.D2/n (H-2^d) (C) B10.BR (H-2^k) or (D) Balb,B (H-2^b) were orally inoculated with 10^7 pfu rotavirus RRV (serotype 3) and 7 days later were sacrificed and the spleens removed. The splenocytes were *in vitro* stimulated to produce the effector cells as described in Figure 25.

The appropriate MHC class I matched target cells [EL4 (H-2^b), P815 (H-2^d) and Ltk$^-$ (H-2^k)] were loaded with ^{51}Cr, infected with WT vaccinia virus or recombinant vaccinia virus (vaccRRV.VP7) and the ^{51}Cr-release assays set up as described in Figure 25.

FIGURE 30


(A) C57BL/10
(H-2b)
(B) B10.D2/n
(H-2d)
% Specific Lysis
35
30
25
20
15
10
5
0
1:1
3:1
10:1
30:1
1:1
3:1
10:1
30:1
Effector:Target Ratio
WT vacc
vaccRRV.VP7
(C) B10.BR
(H-2k)
(D) Balb,B
(H-2b)
% Specific Lysis
20
15
10
5
0
1:1
3:1
10:1
30:1
1:1
3:1
10:1
30:1
Effector:Target Ratio
WT vacc
vaccRRV.VP7

the fundamental factor for the generation of rotavirus RRV VP7-specific CTLs and the response is in no way connected to the minor histocompatibility antigens or any other differences between the various mouse strains used in this experimental work.

8.5 CONCLUSIONS

(i) The major rotavirus UKtc-specific CTL response was generated against the NS53 and VP7 proteins in the mouse H-2^b MHC class I haplotype.

(ii) In the mouse H-2^d and H-2^k MHC class I haplotypes the major rotavirus UKtc-specific CTL response was generated against the VP3 protein.

(iii) A rotavirus RRV VP7-specific CTL response was generated in the mouse H-2^b, but not H-2^d or H-2^k, MHC class I haplotype.

(iv) The mouse strain restriction of the rotavirus RRV VP7-specific CTL response was dependent only on the MHC class I haplotype of the mouse.

(v) A second oral inoculation three weeks after the first, using a different strain of rotavirus at an equal dose (10^7 pfu), was an improved strategy for producing rotavirus-specific CTLs.

CHAPTER 9

THE CTL RESPONSE AGAINST ROTAVIRUS VP3 PROTEIN

9.1 AIMS

Following establishment that the major rotavirus-specific CTL response generated in the mouse H-2^d and H-2^k MHC class I haplotypes was VP3-specific, the next step was to gauge the strain-independence of this response. This is of particular importance because a rotavirus vaccine would need to protect against many of the different rotavirus strains.

9.2 ROTAVIRUS RRV PRIMED CTL RECOGNITION OF TARGET CELLS EXPRESSING HETEROTYPIC ROTAVIRUS UKTC VP3

It has been established that the UKtc NS53 protein did not elicit a specific CTL response in the H-2^d haplotype (S. Stagg, personal communication). Conversely, there is a RRV NS53-specific CTL response in the H-2^d mouse haplotype (as detailed in the Chapter 10) and, therefore, vaccRRV.NS53 infected targets were used as positive controls in the ^{51}Cr-release assays using RRV inoculated H-2^d mice (Figure 31).

No specific CTL response was directed against rotavirus UKtc VP3 protein from rotavirus RRV infected mice of either H-2^d or H-2^k haplotype (see Figures 31 and 32).

9.3 CONCLUSION

A UKtc VP3-specific CTL response was not generated in mice of the H-2^d or H-2^k MHC class I haplotype primed with the RRV strain of rotavirus.

FIGURE 31. % SPECIFIC LYSIS OF TARGETS EXPRESSING UKTC VP3 PROTEIN BY RRV PRIMED BALB/C (H-2^d) EFFECTORS

Two 6 to 10 week old female Balb/c mice (H-2^d) were orally inoculated with 10^7 pfu rotavirus RRV (serotype 3) and 7 days later were sacrificed and the spleens removed. The splenocytes were *in vitro* stimulated to produce the effector cells as described in Figure 25.

P815 target cells (H-2^d) were loaded with ^{51}Cr, infected with WT vaccinia virus or recombinant vaccinia viruses [vaccRRV.NS53 (positive control) or vaccUKtc.VP3 (serotype 6)] and the ^{51}Cr-release assays set up as described in Figure 25.

FIGURE 32. % SPECIFIC LYSIS OF TARGETS EXPRESSING UKTC VP3 PROTEIN BY RRV PRIMED C3H/HE,MG (H-2^k) EFFECTORS

Two 6 to 10 week old female C3H/He,mg mice (H-2^k) were orally inoculated with 10^7 pfu rotavirus RRV (serotype 3) and 7 days later were sacrificed and the spleens removed. The splenocytes were *in vitro* stimulated to produce the effector cells as described in Figure 25.

Ltk$^-$ target cells (H-2^k) were loaded with ^{51}Cr, infected with WT vaccinia virus or recombinant vaccinia virus [vaccUKtc.VP3 (serotype 6)] and the ^{51}Cr-release assays set up as described in Figure 25.

FIGURE 31

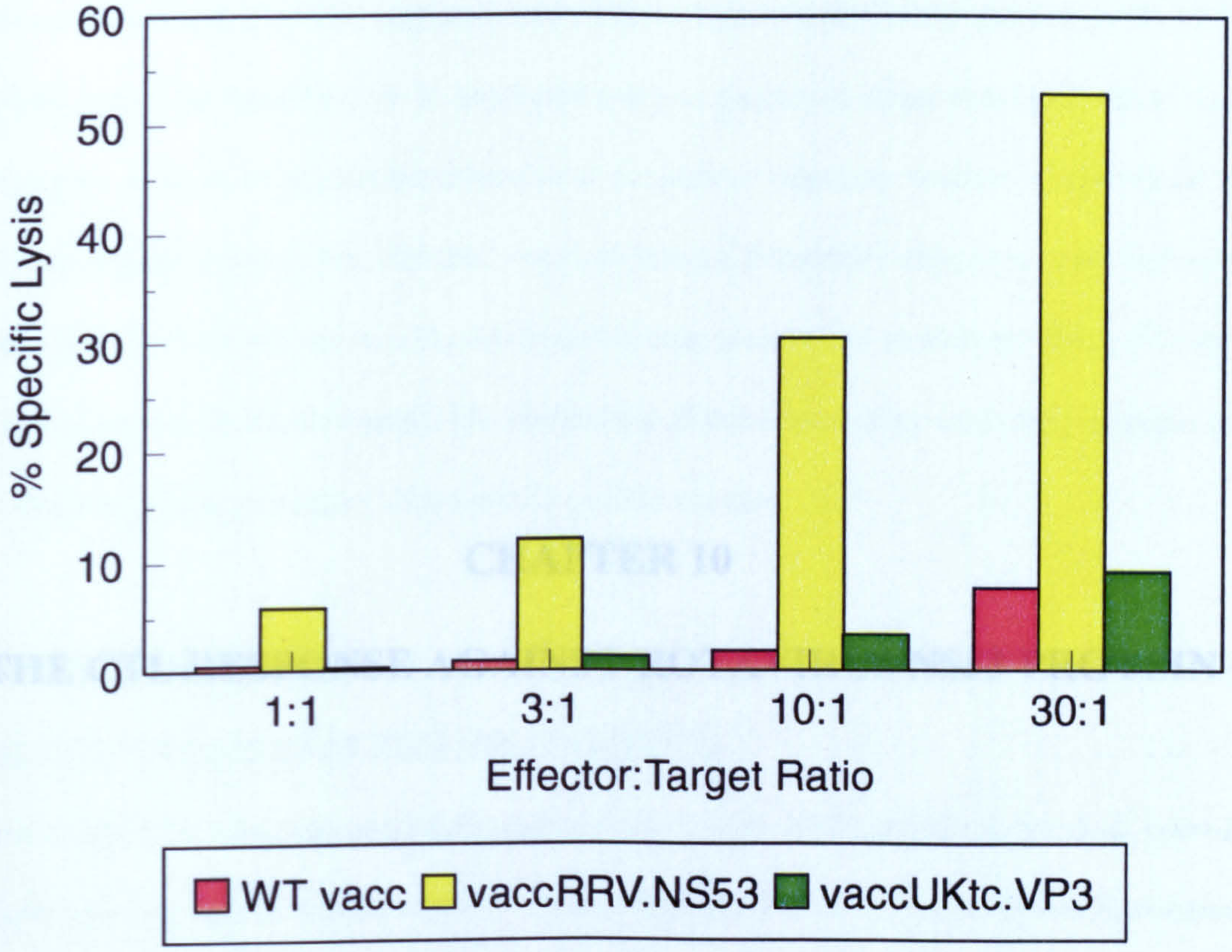
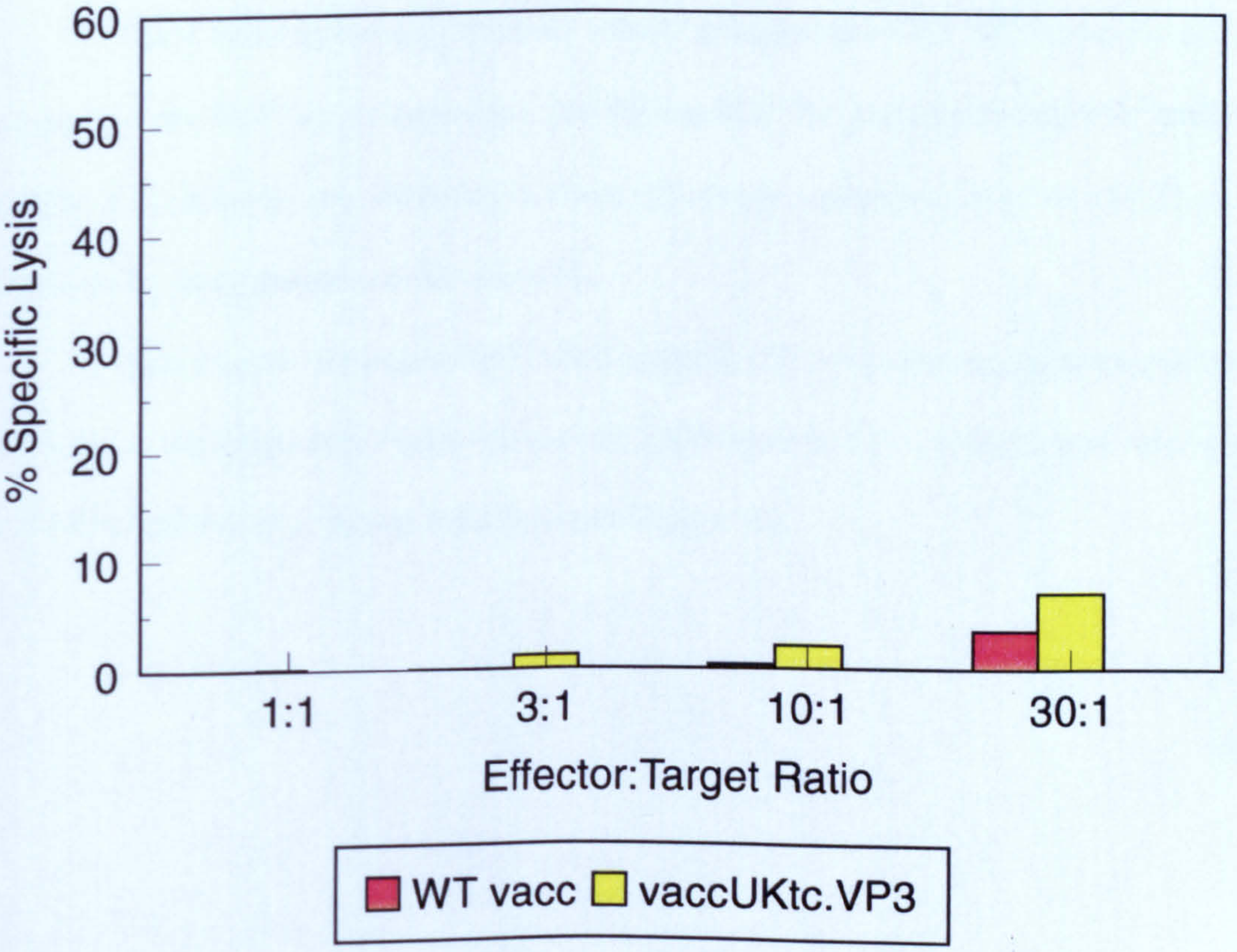


FIGURE 32



CHAPTER 10

THE CTL RESPONSE AGAINST ROTAVIRUS NS53 PROTEIN

10.1 AIMS

The confirmation of a CTL response against the rotavirus UKtc NS53 protein in the H-2^b mouse haplotype (see Chapter 8) prompted work to gauge the strain-independence of this response. This is of particular importance because a rotavirus vaccine would need to protect against many of the different rotavirus strains. Subsidiary objectives were to begin the localisation of the major CTL epitope(s) in this protein and to identify the H-2^b locus of restriction of these epitope(s). The realisation of these secondary aims may be essential in the design of a successful CTL-based rotavirus vaccine.

10.2 CTL RECOGNITION OF TARGET CELLS EXPRESSING VARIOUS HETEROTYPIC ROTAVIRUS NS53 PROTEINS

The major CTL response generated against the rotavirus NS53 protein in the H-2^b mouse haplotype was further examined for a strain-independent CTL response. It was found that the CTL response to rotavirus NS53 was strain-dependent in the H-2^b mouse haplotype, regardless of the inoculating rotavirus strain (UKtc or RRV) (see Figures 33 and 34).

These experiments did, however, reveal a homotypic RRV NS53-specific CTL response in the H-2^b mouse haplotype (see Figure 34). The analysis of the RRV NS53-specific CTL response was extended to cover, (i) mouse haplotypes other than H-2^b, and (ii) the strain-independence of this response.

Only a strain-dependent RRV NS53-specific CTL response was generated in the H-2^d mouse haplotype (see Figure 35) and no NS53-specific CTL response was observed in the RRV primed H-2^k mouse haplotype (see Figure 36).

FIGURE 33. % SPECIFIC LYSIS OF TARGETS EXPRESSING VARIOUS NS53 PROTEINS BY UKTC PRIMED C57BL/6 (H-2^b) EFFECTORS

Two 6 to 10 week old female C57BL/6 mice (H-2^b) were orally inoculated with 10^7 pfu rotavirus UKtc (serotype 6) and 7 days later were sacrificed and the spleens removed. The splenocytes were *in vitro* stimulated to produce the effector cells as described in Figure 25.

EL4 target cells (H-2^b) were loaded with ^{51}Cr, infected with WT vaccinia virus or recombinant vaccinia viruses [vaccUKtc.NS53 (positive control), vaccRRV.NS53 (serotype 3) or vaccHochi.NS53 (serotype 4)] and the ^{51}Cr-release assays set up as described in Figure 25.

FIGURE 34. % SPECIFIC LYSIS OF TARGETS EXPRESSING VARIOUS NS53 PROTEINS BY RRV PRIMED C57BL/6 (H-2^b) EFFECTORS

Two 6 to 10 week old female C57BL/6 mice (H-2^b) were orally inoculated with 10^7 pfu rotavirus RRV (serotype 3) and 7 days later were sacrificed and the spleens removed. The splenocytes were *in vitro* stimulated to produce the effector cells as described in Figure 25.

EL4 target cells (H-2^b) were loaded with ^{51}Cr, infected with WT vaccinia virus or recombinant vaccinia viruses [vaccUKtc.NS53 (serotype 6), vaccRRV.NS53 (positive control) or vaccHochi.NS53 (serotype 4)] and the ^{51}Cr-release assays set up as described in Figure 25.

FIGURE 33

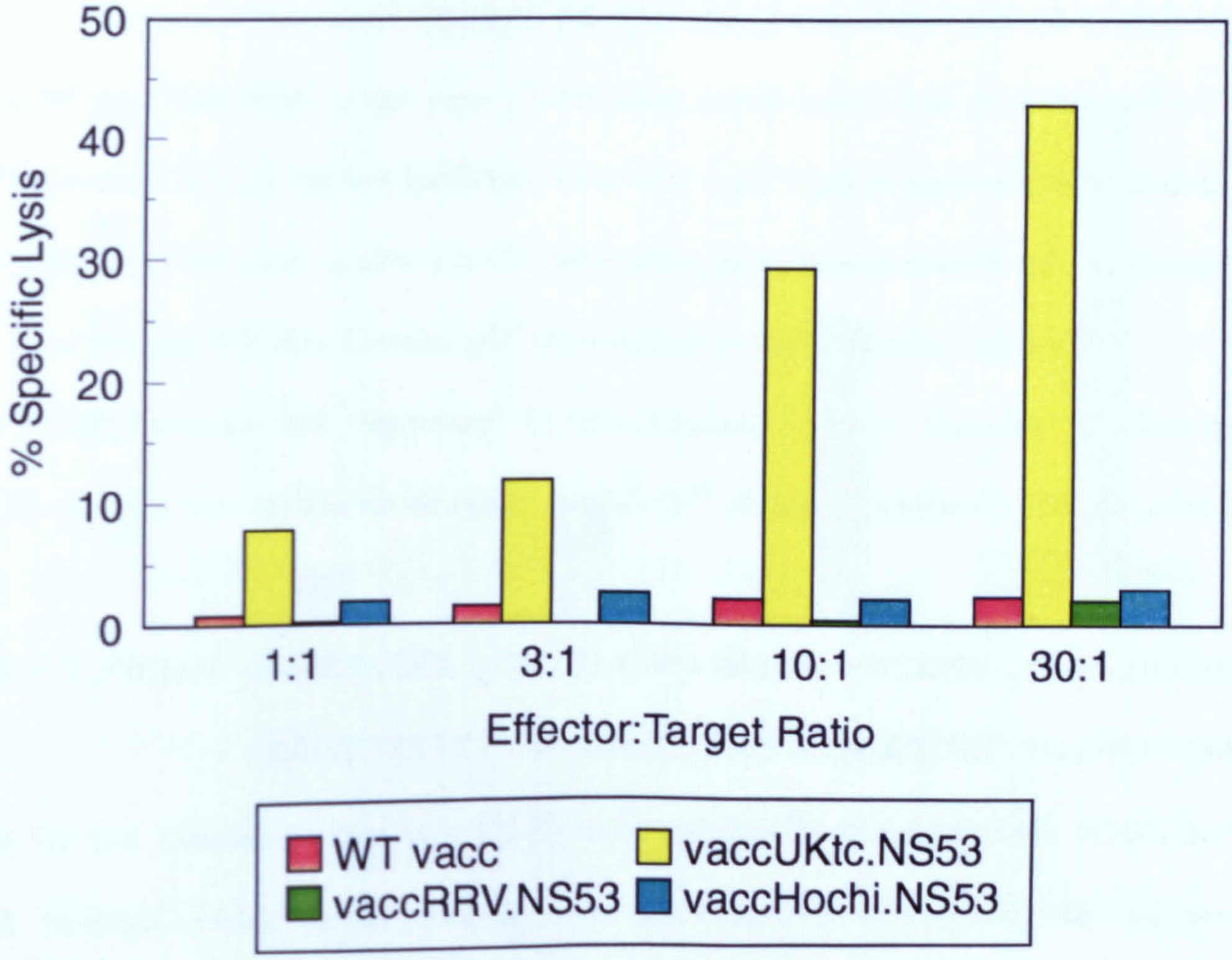


FIGURE 34

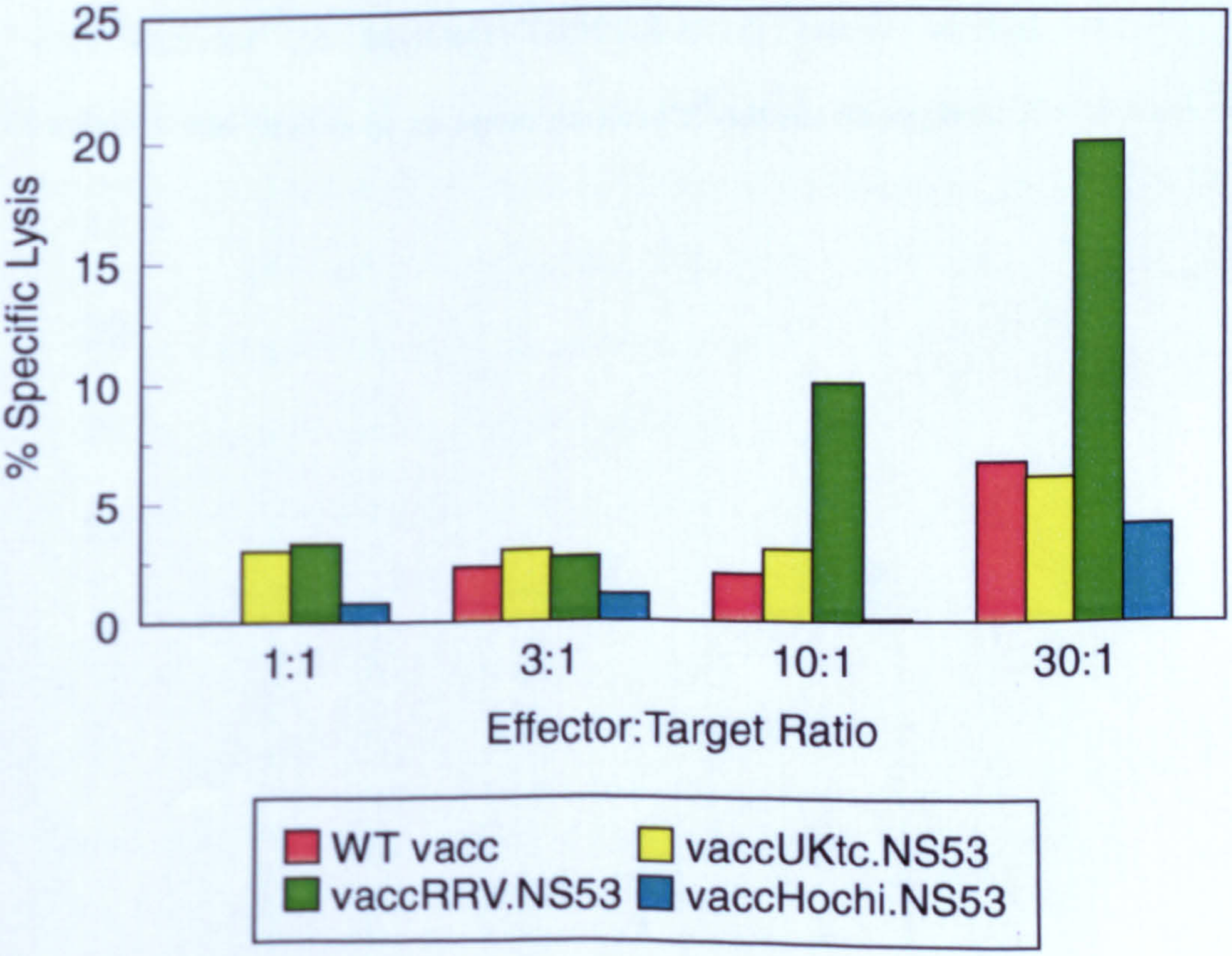


FIGURE 35. % SPECIFIC LYSIS OF TARGETS EXPRESSING VARIOUS NS53 PROTEINS BY RRV PRIMED BALB/C (H-2^d) EFFECTORS

Two 6 to 10 week old female Balb/c mice (H-2^d) were orally inoculated with 10^7 pfu rotavirus RRV (serotype 3) and 7 days later were sacrificed and the spleens removed. The splenocytes were *in vitro* stimulated to produce the effector cells as described in Figure 25.

P815 target cells (H-2^d) were loaded with ^{51}Cr, infected with WT vaccinia virus or recombinant vaccinia viruses [vaccUKtc.NS53 (serotype 6), vaccRRV.NS53 or vaccHochi.NS53 (serotype 4)] and the ^{51}Cr-release assays set up as described in Figure 25.

FIGURE 36. % SPECIFIC LYSIS OF TARGETS EXPRESSING VARIOUS NS53 PROTEINS BY RRV PRIMED C3H/HE,MG (H-2^k) EFFECTORS

Two 6 to 10 week old female C3H/He,mg mice (H-2^k) were orally inoculated with 10^7 pfu rotavirus RRV (serotype 3) and 7 days later were sacrificed and the spleens removed. The splenocytes were *in vitro* stimulated to produce the effector cells as described in Figure 25.

Ltk$^-$ target cells (H-2^k) were loaded with ^{51}Cr, infected with WT vaccinia virus or recombinant vaccinia viruses [vaccUKtc.NS53 (serotype 6), vaccRRV.NS53 or vaccHochi.NS53 (serotype 4)] and the ^{51}Cr-release assays set up as described in Figure 25.

FIGURE 35

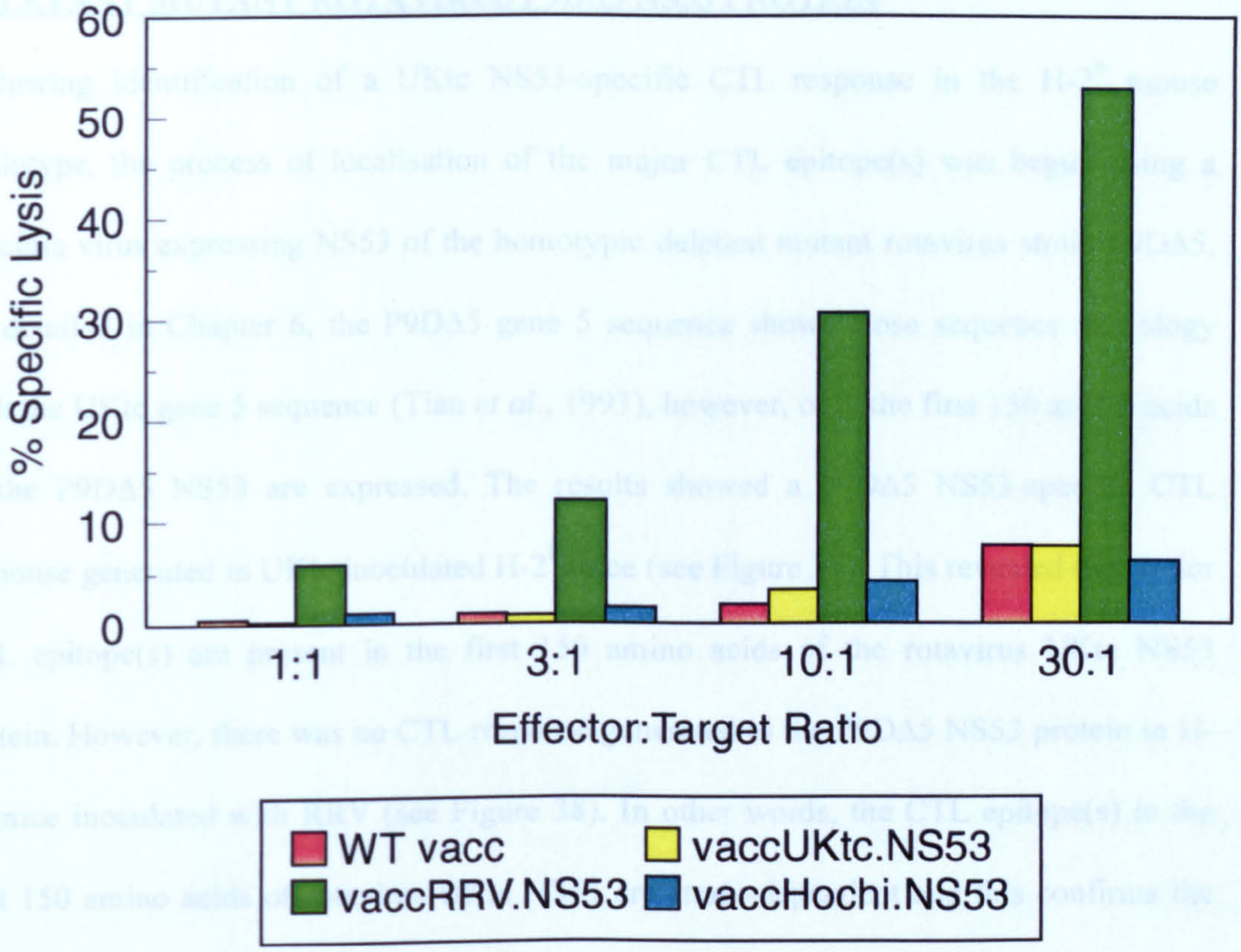
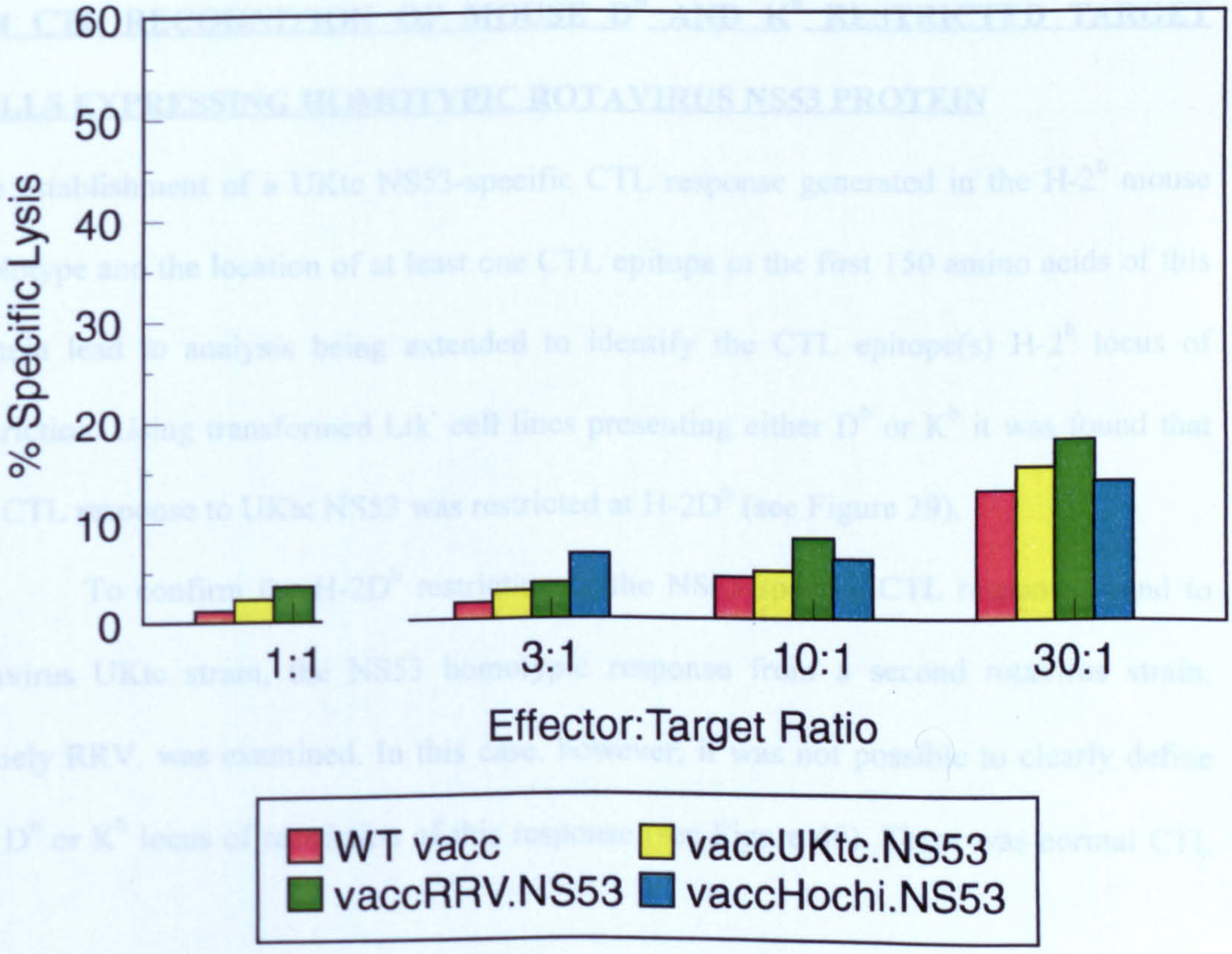


FIGURE 36



10.3 CTL RECOGNITION OF H-2^b TARGET CELLS EXPRESSING THE DELETANT MUTANT ROTAVIRUS P9DΔ5 NS53 PROTEIN

Following identification of a UKtc NS53-specific CTL response in the H-2^b mouse haplotype, the process of localisation of the major CTL epitope(s) was begun using a vaccinia virus expressing NS53 of the homotypic deletant mutant rotavirus strain P9DΔ5. As detailed in Chapter 6, the P9DΔ5 gene 5 sequence shows close sequence homology with the UKtc gene 5 sequence (Tian *et al.*, 1993), however, only the first 150 amino acids of the P9DΔ5 NS53 are expressed. The results showed a P9DΔ5 NS53-specific CTL response generated in UKtc inoculated H-2^b mice (see Figure 37). This revealed that major CTL epitope(s) are present in the first 150 amino acids of the rotavirus UKtc NS53 protein. However, there was no CTL response generated to the P9DΔ5 NS53 protein in H-2^b mice inoculated with RRV (see Figure 38). In other words, the CTL epitope(s) in the first 150 amino acids of rotavirus UKtc NS53 are strain-dependent and this confirms the homotypic nature of the NS53-specific CTL response.

10.4 CTL RECOGNITION OF MOUSE D^b AND K^b RESTRICTED TARGET CELLS EXPRESSING HOMOTYPIC ROTAVIRUS NS53 PROTEIN

The establishment of a UKtc NS53-specific CTL response generated in the H-2^b mouse haplotype and the location of at least one CTL epitope in the first 150 amino acids of this protein lead to analysis being extended to identify the CTL epitope(s) H-2^b locus of restriction. Using transformed Ltk$^-$ cell lines presenting either D^b or K^b it was found that the CTL response to UKtc NS53 was restricted at H-2D^b (see Figure 39).

To confirm the H-2D^b restriction of the NS53-specific CTL response found to rotavirus UKtc strain, the NS53 homotypic response from a second rotavirus strain, namely RRV, was examined. In this case, however, it was not possible to clearly define the D^b or K^b locus of restriction of this response (see Figure 40). There was normal CTL

FIGURE 37. % SPECIFIC LYSIS OF TARGETS EXPRESSING P9DΔ5 NS53 PROTEIN BY UKTC PRIMED C57BL/6 (H-2^b) EFFECTORS

Two 6 to 10 week old female C57BL/6 mice (H-2^b) were orally inoculated with 10^7 pfu rotavirus UKtc (serotype 6) and 7 days later were sacrificed and the spleens removed. The splenocytes were *in vitro* stimulated to produce the effector cells as described in Figure 25.

EL4 target cells (H-2^b) were loaded with ^{51}Cr, infected with WT vaccinia virus or recombinant vaccinia virus [vaccP9DΔ5.NS53 (serotype 6)] and the ^{51}Cr-release assays set up as described in Figure 25.

FIGURE 38. % SPECIFIC LYSIS OF TARGETS EXPRESSING P9DΔ5 NS53 PROTEIN BY RRV PRIMED C57BL/6 (H-2^b) EFFECTORS

Two 6 to 10 week old female C57BL/6 mice (H-2^b) were orally inoculated with 10^7 pfu rotavirus RRV (serotype 3) and 7 days later were sacrificed and the spleens removed. The splenocytes were *in vitro* stimulated to produce the effector cells as described in Figure 25.

EL4 target cells (H-2^b) were loaded with ^{51}Cr, infected with WT vaccinia virus or recombinant vaccinia viruses [vaccRRV.NS53 (positive control) or vaccP9DΔ5.NS53 (serotype 6)] and the ^{51}Cr-release assays set up as described in Figure 25.

FIGURE 37

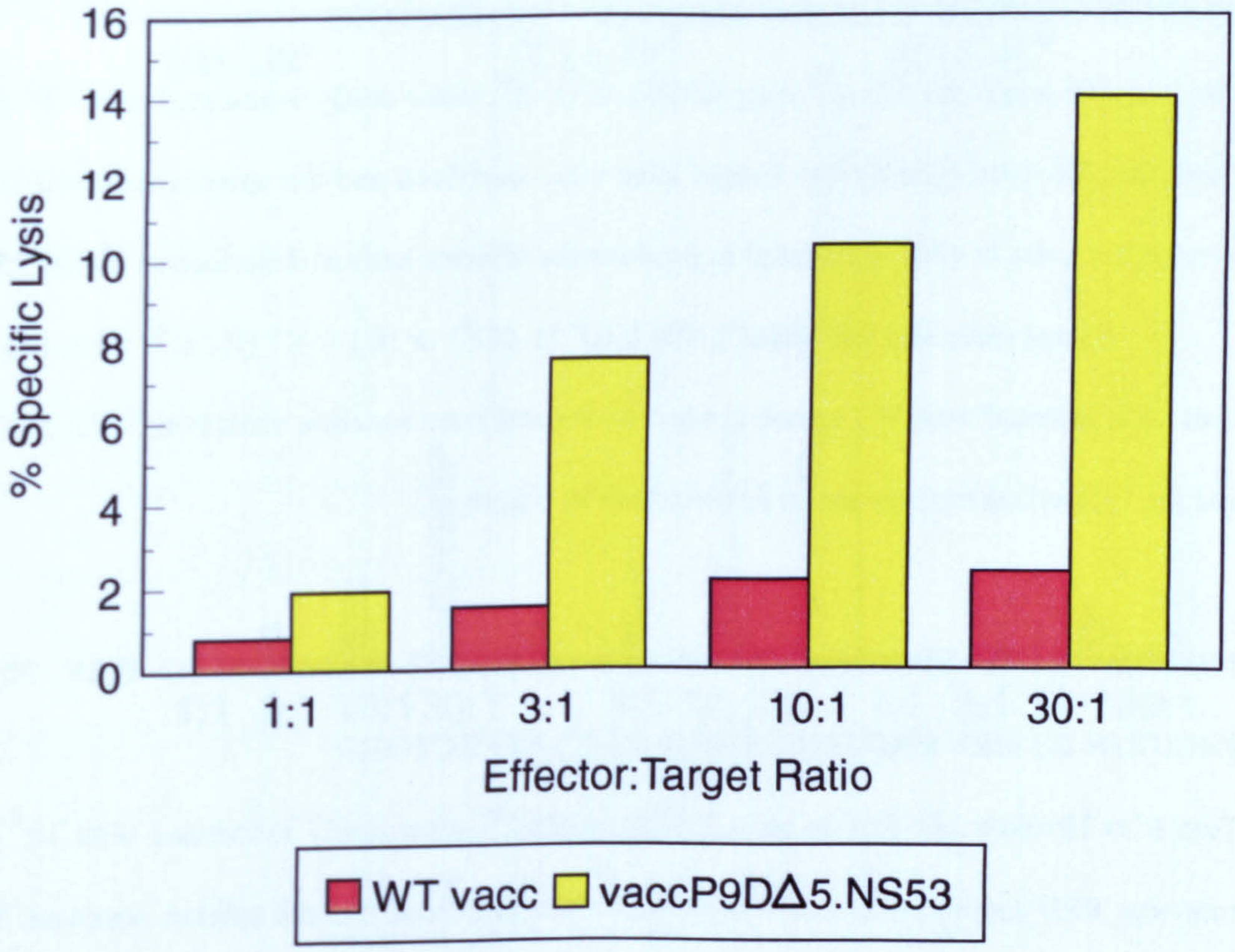


FIGURE 38

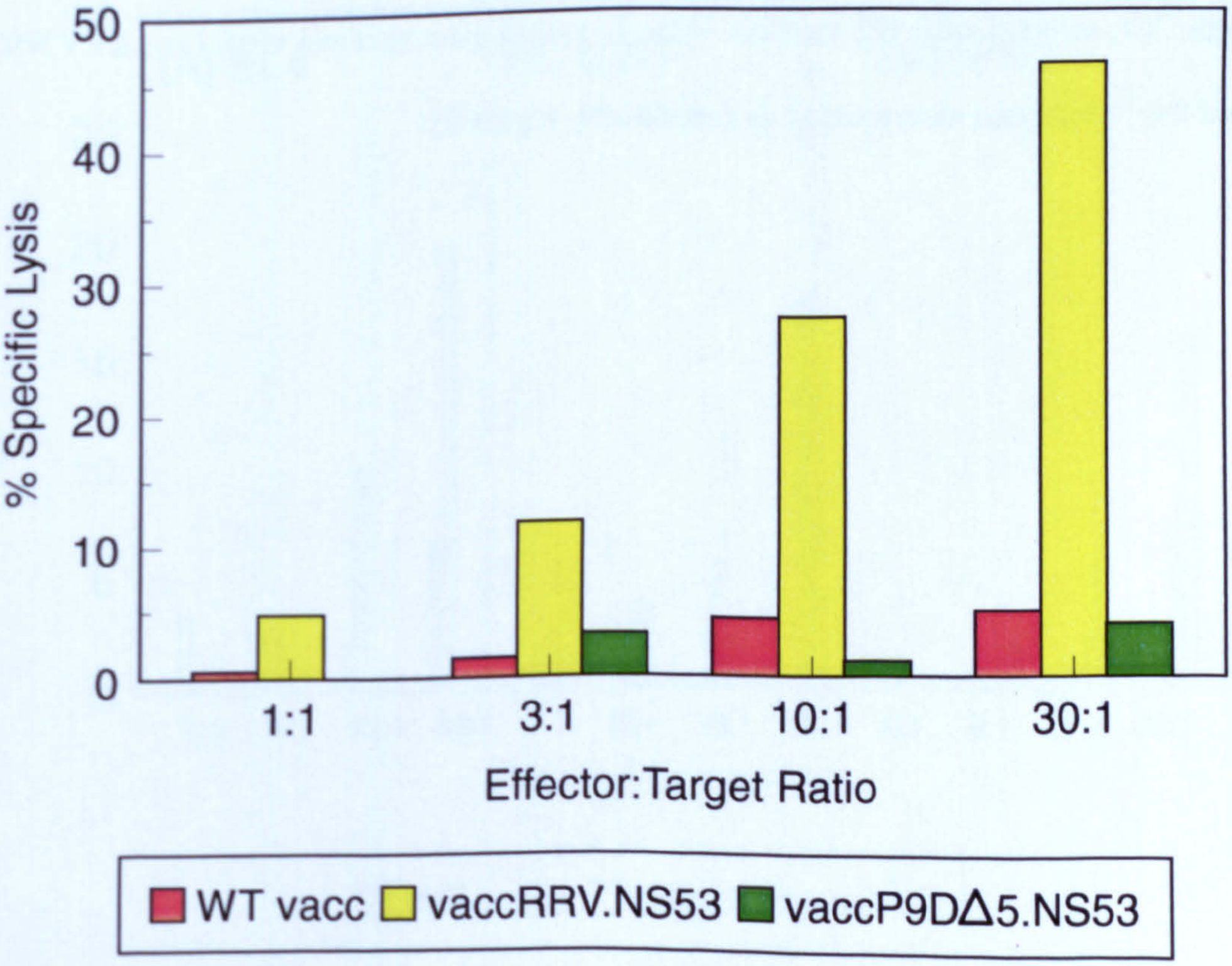


FIGURE 39. % SPECIFIC LYSIS OF TARGETS EXPRESSING UKTC NS53 PROTEIN BY UKTC PRIMED C57BL/6 (H-2^b) EFFECTORS

Two 6 to 10 week old female mice C57BL/6 (H-2^b) were orally inoculated with 10^7 pfu rotavirus UKtc (serotype 6) and 7 days later were sacrificed and the spleens removed. The splenocytes were *in vitro* stimulated to produce the effector cells as described in Figure 25.

Target cells (A) Ltk$^-$ (H-2^k), (B) L-D^b (L-2D^b) or (C) L-K^b (H-2K^b) were loaded with ^{51}Cr, infected with WT vaccinia virus or recombinant vaccinia virus (vaccUKtc.NS53) and the ^{51}Cr-release assays set up as described in Figure 25.

FIGURE 40. % SPECIFIC LYSIS OF TARGETS EXPRESSING RRV NS53 PROTEIN BY RRV PRIMED C57BL/6 (H-2^b) EFFECTORS

Two 6 to 10 week old female mice C57BL/6 (H-2^b) were orally inoculated with 10^7 pfu rotavirus RRV (serotype 3) and 7 days later were sacrificed and the spleens removed. The splenocytes were *in vitro* stimulated to produce the effector cells as described in Figure 25.

Target cells (A) EL4 (H-2^b), (B) L-D^b (L-2D^b) or (C) L-K^b (H-2K^b) were loaded with ^{51}Cr, infected with WT vaccinia virus or recombinant vaccinia virus (vaccRRV.NS53) and the ^{51}Cr-release assays set up as described in Figure 25.

FIGURE 39

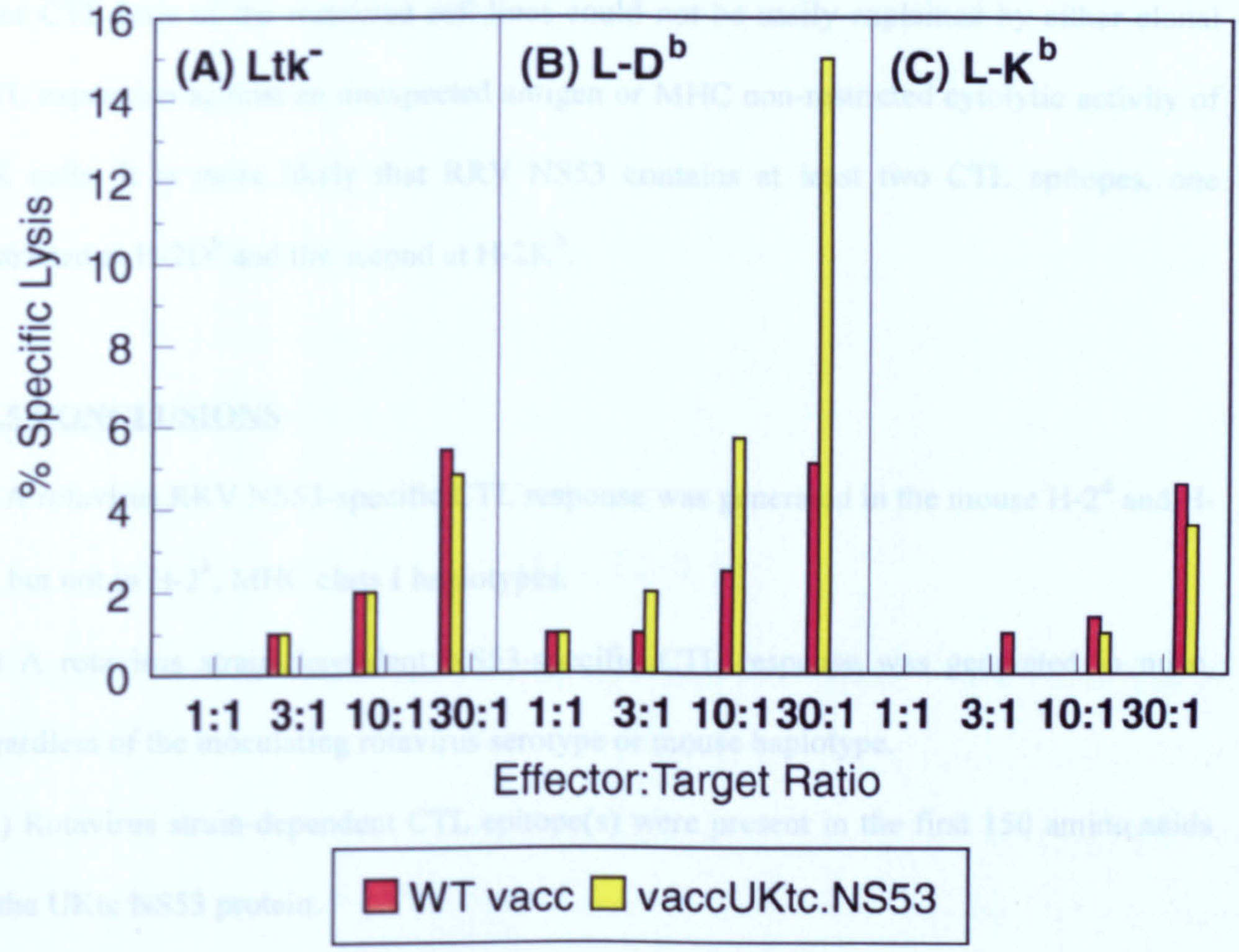
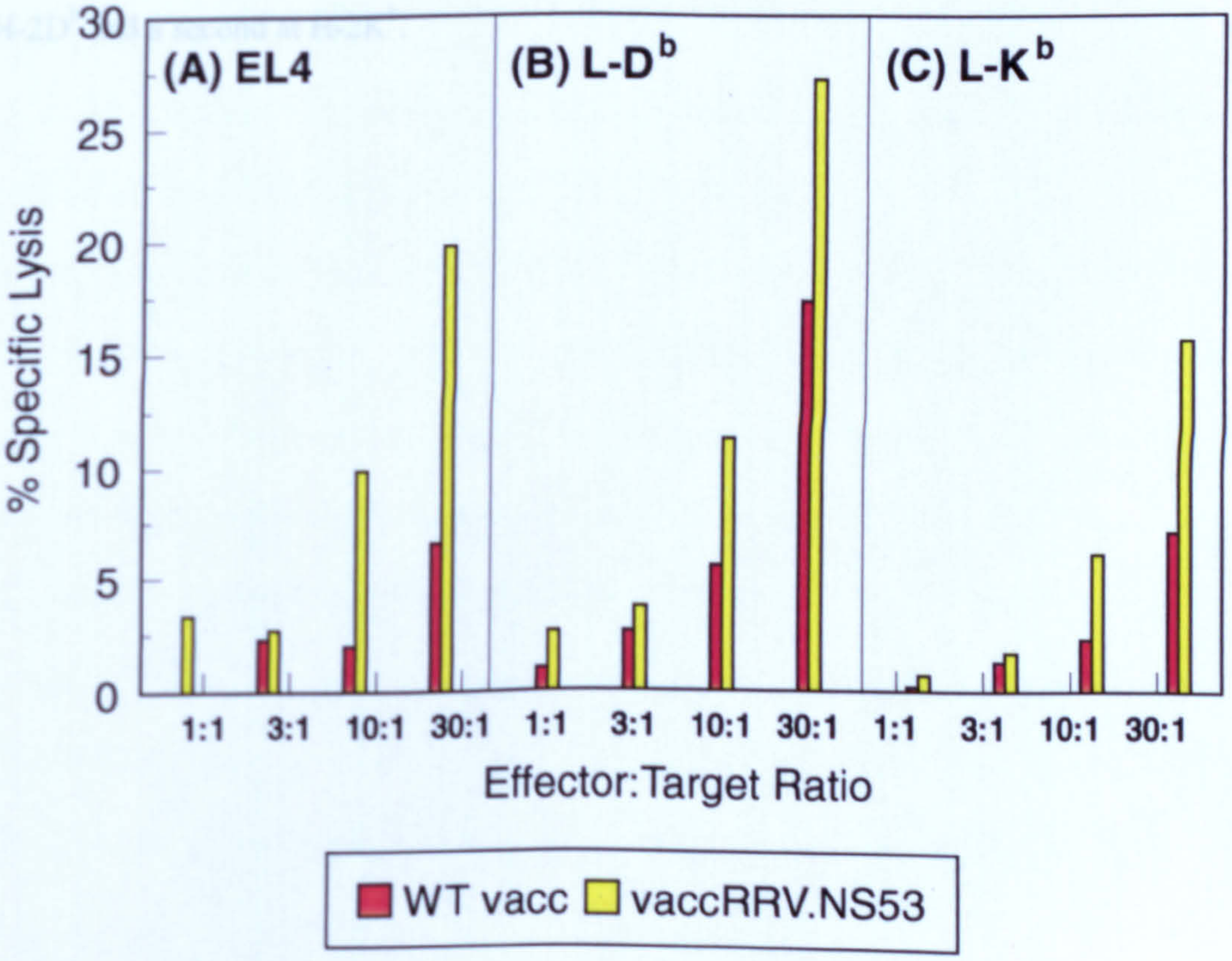


FIGURE 40



lysis of the positive target cell line (H-2^b) infected with vaccRRV.NS53 and, therefore, the poor CTL lysis of the restricted cell lines could not be easily explained by either clonal CTL expansion against an unexpected antigen or MHC non-restricted cytolytic activity of NK cells. It is more likely that RRV NS53 contains at least two CTL epitopes, one restricted at H-$2D^b$ and the second at H-$2K^b$.

10.5 CONCLUSIONS

(i) A rotavirus RRV NS53-specific CTL response was generated in the mouse H-2^d and H-2^b, but not in H-2^k, MHC class I haplotypes.

(ii) A rotavirus strain-dependent NS53-specific CTL response was generated in mice, regardless of the inoculating rotavirus serotype or mouse haplotype.

(iii) Rotavirus strain-dependent CTL epitope(s) were present in the first 150 amino acids of the UKtc NS53 protein.

(iv) The UKtc NS53-specific CTL response was restricted at H-$2D^b$, whereas, the RRV NS53-specific CTL response probably involved at least two CTL epitopes, one restricted at H-$2D^b$ and a second at H-$2K^b$.

CHAPTER 11

THE CTL RESPONSE AGAINST ROTAVIRUS VP7 PROTEIN

11.1 AIMS

Following confirmation of the rotavirus UKtc VP7-specific CTL response in the H-2^b mouse haplotype (see Chapter 8), the next step was to gauge the serotype-independence of this response. This is of particular importance because a rotavirus vaccine would need to protect against many of the different rotavirus serotypes. A second objective was to begin the localisation of the major CTL epitope(s) in this protein and to identify the H-2^b locus of restriction of these epitope(s). This localisation and classification of epitopes may be essential in the design of a successful CTL-based rotavirus vaccine.

11.2 CTL RECOGNITION OF TARGET CELLS EXPRESSING VARIOUS HETEROTYPIC ROTAVIRUS VP7 PROTEINS

The major CTL response generated against the rotavirus VP7 protein in the H-2^b mouse haplotype was further examined for a serotype-independent CTL response. It was found that the CTL response was serotype-independent across seven different rotavirus VP7 serotypes, regardless of the inoculating serotype (3 or 6) (see Figures 41 and 42).

11.3 CTL RECOGNITION OF H-2^b TARGET CELLS EXPRESSING FRAGMENTS OF ROTAVIRUS UKTC VP7 PROTEIN

After establishment of a UKtc VP7-specific CTL response in the H-2^b mouse haplotype, the localisation of the CTL epitope(s) was performed using target cells expressing restriction enzyme fragments of UKtc VP7 protein (see Figure 20). There was only a VP7(ClaI-HhaI)-specific CTL response generated in UKtc inoculated H-2^b mice (see Figure 43). This reveals that CTL epitope(s) are present between amino acids 13 and 48 of the rotavirus UKtc VP7 protein. However, there was no CTL response generated to the VP7(ClaI-HhaI) fragment in H-2^b mice inoculated with RRV virus (see Figure 44), in other words, the CTL epitope(s) between amino acids 13 and 48 of the UKtc VP7 protein

FIGURE 41. % SPECIFIC LYSIS OF TARGETS EXPRESSING VARIOUS VP7 PROTEINS BY UKTC PRIMED C57BL/6 (H-2^b) EFFECTORS

Two 6 to 10 week old female C57BL/6 mice (H-2^b) were orally inoculated with 10^7 pfu rotavirus UKtc (serotype 6) and 7 days later were sacrificed and the spleens removed. The splenocytes were *in vitro* stimulated to produce the effector cells as described in Figure 25.

EL4 target cells (H-2^b) were loaded with ^{51}Cr, infected with WT vaccinia virus or recombinant vaccinia viruses [vaccWa.VP7 (serotype 1), vaccRRV.VP7 (serotype 3), vaccHochi.VP7 (serotype 4), vaccUKtc.VP7 (serotype 6), vacc69M.VP7 (serotype 8), vaccWI-61.VP7 (serotype 9) or vaccA64.VP7 (serotype 10)] and the ^{51}Cr-release assays set up as described in Figure 25.

FIGURE 42. % SPECIFIC LYSIS OF TARGETS EXPRESSING VARIOUS VP7 PROTEINS BY RRV PRIMED C57BL/6 (H-2^b) EFFECTORS

Two 6 to 10 week old female C57BL/6 mice (H-2^b) were orally inoculated with 10^7 pfu rotavirus RRV (serotype 3) and 7 days later were sacrificed and the spleens removed. The splenocytes were *in vitro* stimulated to produce the effector cells as described in Figure 25.

EL4 target cells (H-2^b) were loaded with ^{51}Cr, infected with WT vaccinia virus or recombinant vaccinia viruses [vaccWa.VP7 (serotype 1), vaccRRV.VP7 (serotype 3), vaccHochi.VP7 (serotype 4), vaccUKtc.VP7 (serotype 6), vacc69M.VP7 (serotype 8), vaccWI-61.VP7 (serotype 9) or vaccA64.VP7 (serotype 10)] and the ^{51}Cr-release assays set up as described in Figure 25.

FIGURE 41

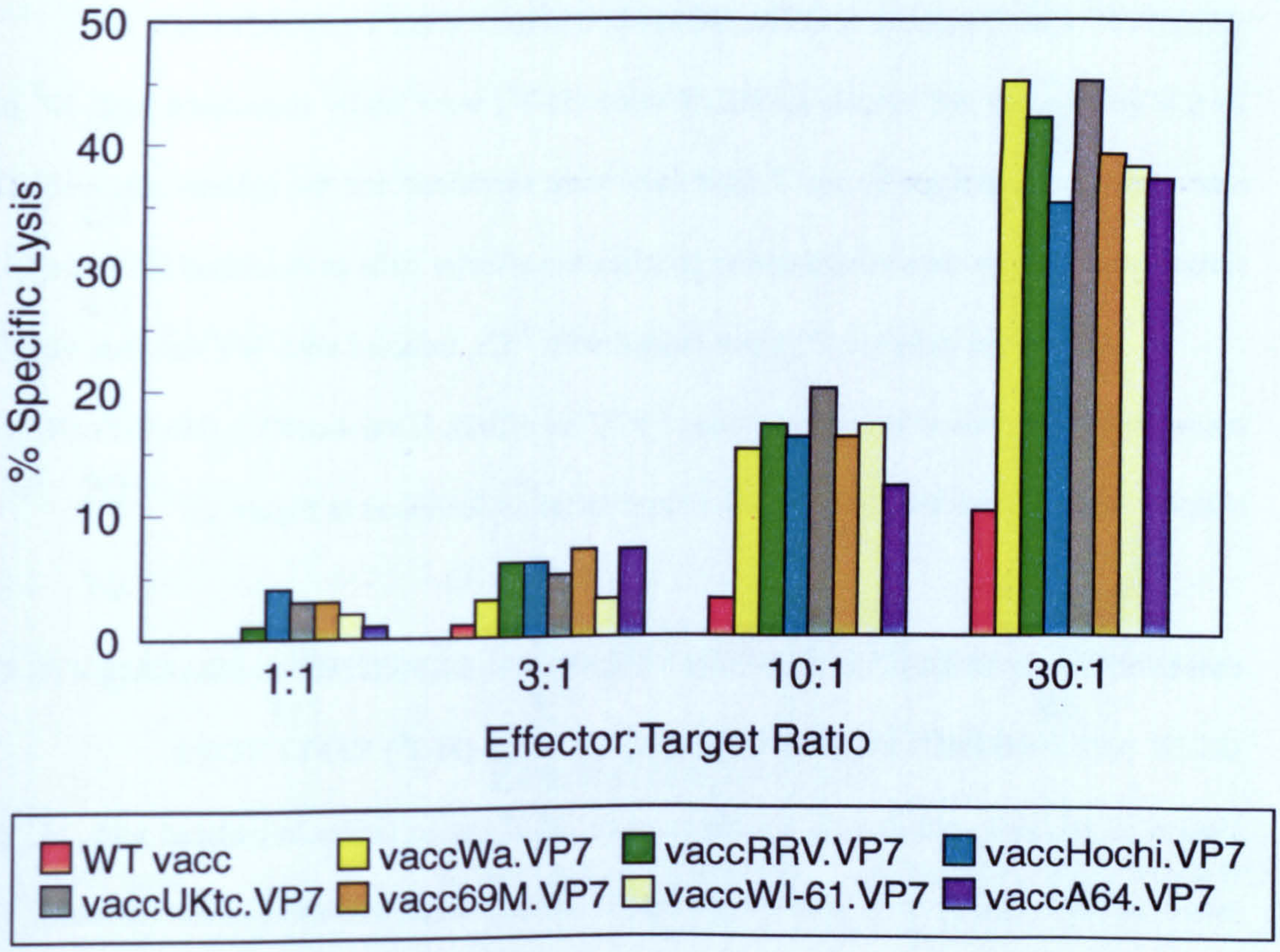


FIGURE 42

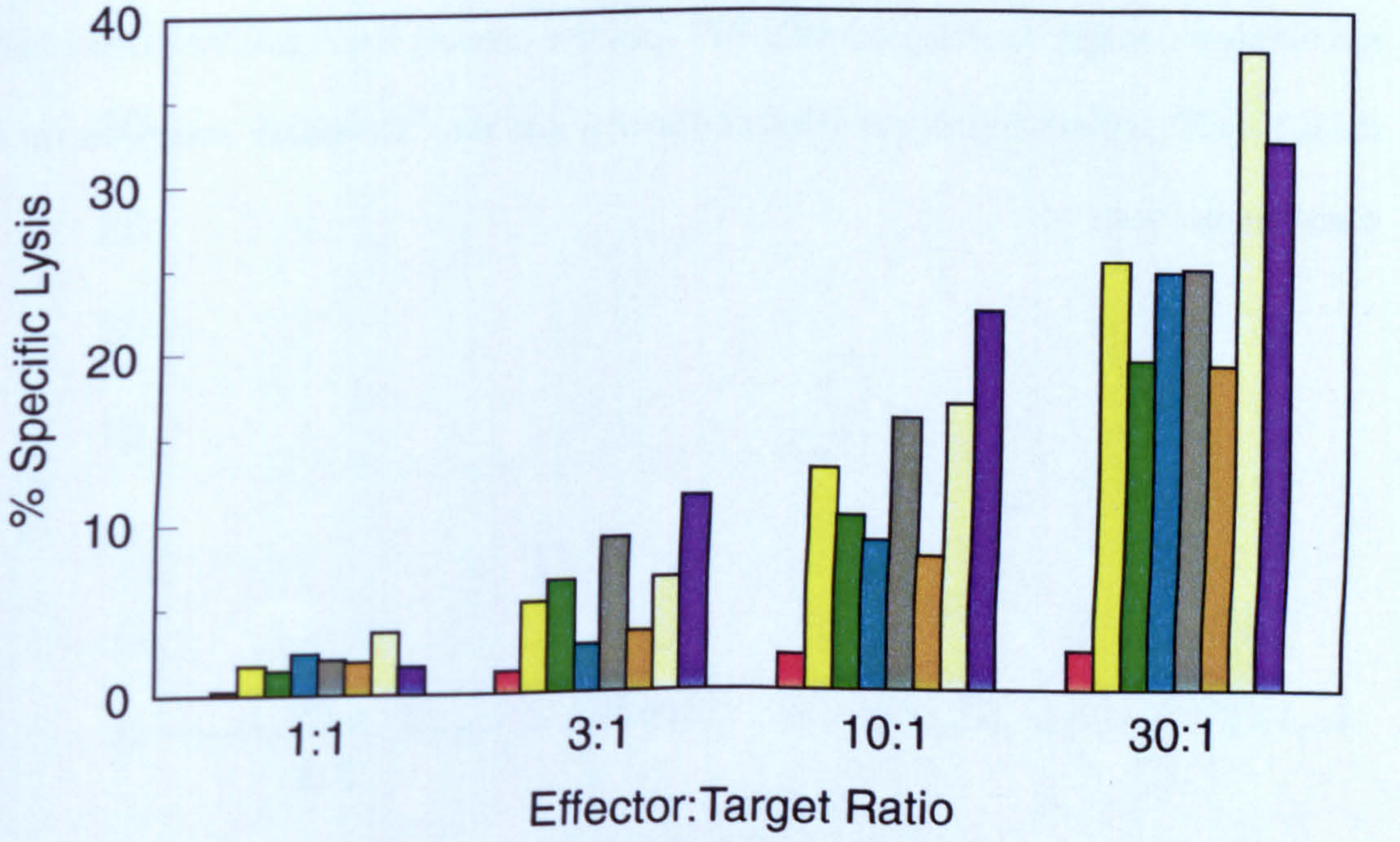


FIGURE 43. % SPECIFIC LYSIS OF TARGETS EXPRESSING FRAGMENTS OF UKTC VP7 PROTEIN BY UKTC PRIMED C57BL/6 (H-2^b) EFFECTORS

Two 6 to 10 week old female C57BL/6 mice (H-2^b) were orally inoculated with 10^7 pfu rotavirus UKtc (serotype 6) and 7 days later were sacrificed and the spleens removed. The splenocytes were *in vitro* stimulated to produce the effector cells as described in Figure 25.

EL4 target cells (H-2^b) were loaded with ^{51}Cr, infected with WT vaccinia virus or recombinant vaccinia viruses [vaccUKtc.VP7(ClaI-HhaI), (DraI-EcoRV), (HinfI-EcoRV) or (EcoRV-HincII)] and the ^{51}Cr-release assays set up as described in Figure 25.

FIGURE 44. % SPECIFIC LYSIS OF TARGETS EXPRESSING FRAGMENTS OF UKTC VP7 PROTEIN BY RRV PRIMED C57BL/6 (H-2^b) EFFECTORS

Two 6 to 10 week old female C57BL/6 mice (H-2^b) were orally inoculated with 10^7 pfu rotavirus RRV (serotype 3) and 7 days later were sacrificed and the spleens removed. The splenocytes were *in vitro* stimulated to produce the effector cells as described in Figure 25.

EL4 target cells (H-2^b) were loaded with ^{51}Cr, infected with WT vaccinia virus or recombinant vaccinia viruses [vaccRRV.VP7 (positive control), vaccUKtc.VP7(ClaI-HhaI), (DraI-EcoRV), (HinfI-EcoRV) or (EcoRV-HincII)] and the ^{51}Cr-release assays set up as described in Figure 25.

FIGURE 43

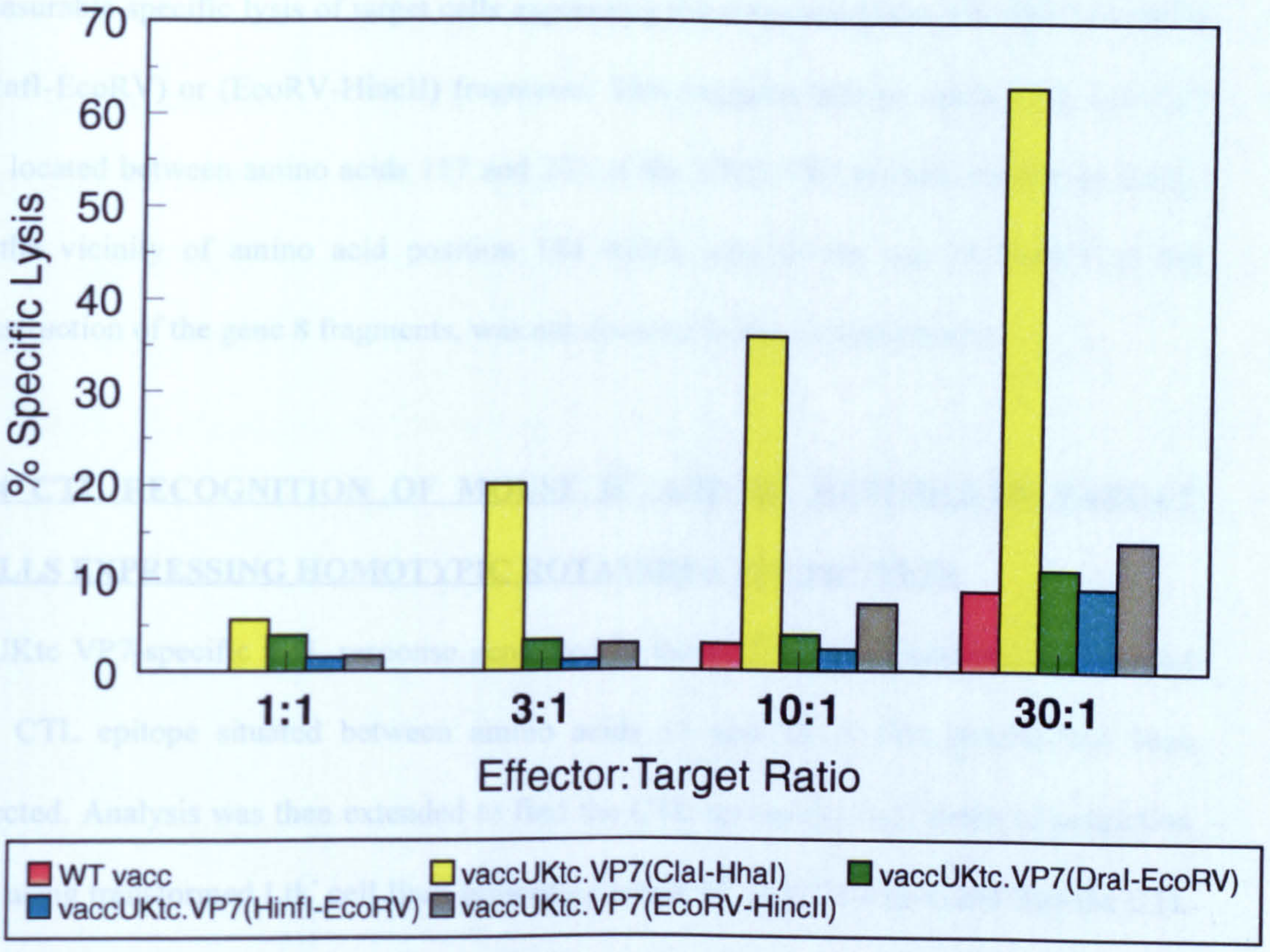
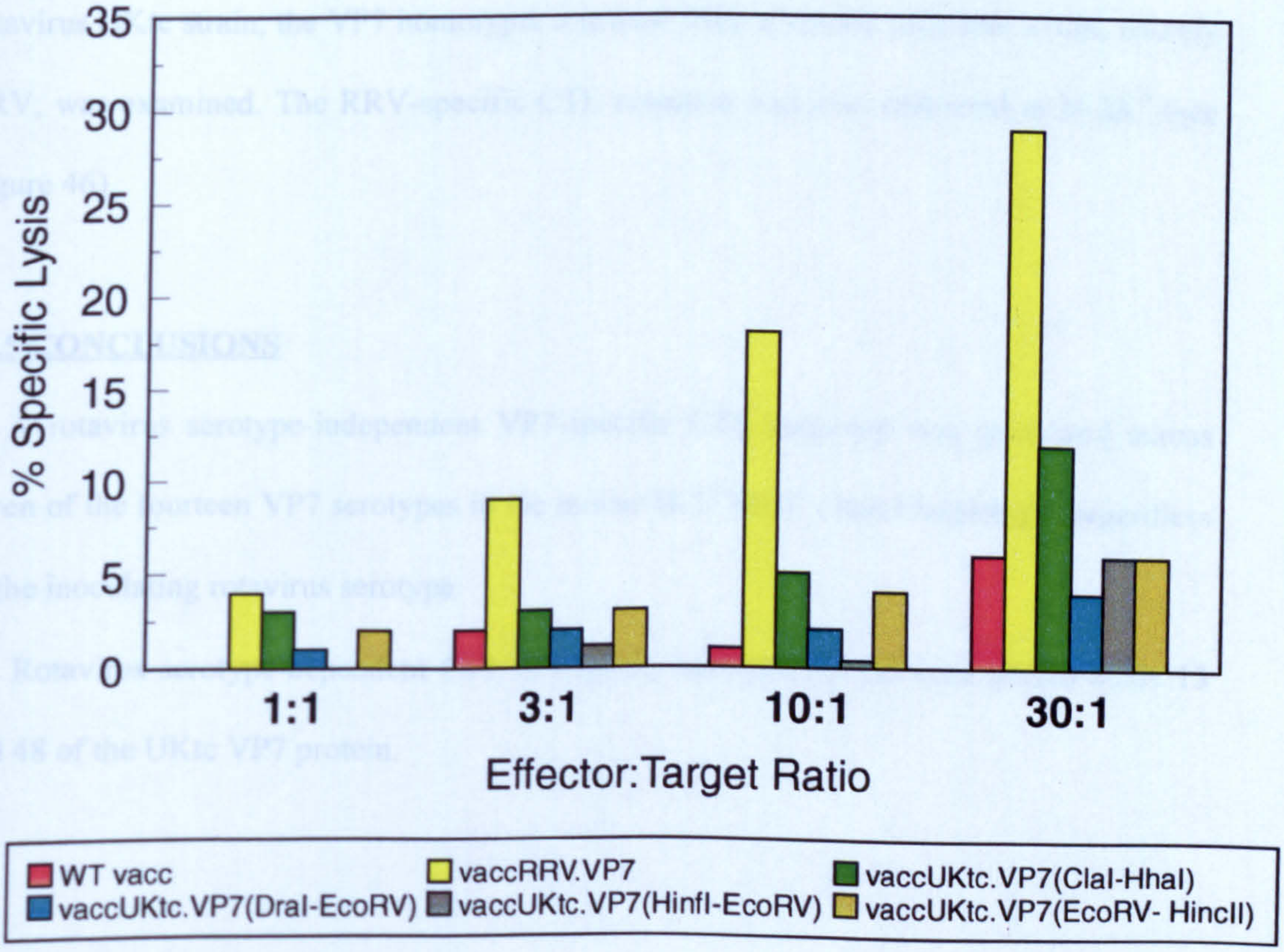


FIGURE 44



are not the serotype-independent epitope(s) of this protein. In addition, there was no measurable specific lysis of target cells expressing the rotavirus UKtc VP7(DraI-EcoRV), (HinfI-EcoRV) or (EcoRV-HincII) fragments. This suggests that no major CTL epitopes are located between amino acids 117 and 232 of the UKtc VP7 protein (except, possibly, in the vicinity of amino acid position 184 which, due to the use of EcoRV in the construction of the gene 8 fragments, was not covered in these experiments).

11.4 CTL RECOGNITION OF MOUSE D^b AND K^b RESTRICTED TARGET CELLS EXPRESSING HOMOTYPIC ROTAVIRUS VP7 PROTEIN

A UKtc VP7-specific CTL response generated in the H-2^b mouse haplotype and at least one CTL epitope situated between amino acids 13 and 48 of this protein had been detected. Analysis was then extended to find the CTL epitope(s) H-2^b locus of restriction and using transformed Ltk^- cell lines presenting either D^b or K^b it was found that the CTL response to UKtc VP7 was restricted at H-$2K^b$ (see Figure 45).

To confirm the H-$2K^b$ restriction of the VP7-specific CTL response found to rotavirus UKtc strain, the VP7 homotypic response from a second rotavirus strain, namely RRV, was examined. The RRV-specific CTL response was also restricted at H-$2K^b$ (see Figure 46).

11.5 CONCLUSIONS

(i) A rotavirus serotype-independent VP7-specific CTL response was generated across seven of the fourteen VP7 serotypes in the mouse H-2^b MHC class I haplotype, regardless of the inoculating rotavirus serotype.

(ii) Rotavirus serotype-dependent CTL epitope(s) were present between amino acids 13 and 48 of the UKtc VP7 protein.

FIGURE 45. % SPECIFIC LYSIS OF TARGETS EXPRESSING UKTC VP7 PROTEIN BY UKTC PRIMED C57BL/6 (H-2^b) EFFECTORS

Two 6 to 10 week old female mice C57BL/6 (H-2^b) were orally inoculated with 10^7 pfu rotavirus UKtc (serotype 6) and 7 days later were sacrificed and the spleens removed. The splenocytes were *in vitro* stimulated to produce the effector cells as described in Figure 25.

Target cells (A) EL4 (H-2^b), (B) L-D^b (L-$2D^b$) or (C) L-K^b (H-$2K^b$) were loaded with ^{51}Cr, infected with WT vaccinia virus or recombinant vaccinia virus (vaccUKtc.VP7) and the ^{51}Cr-release assays set up as described in Figure 25.

FIGURE 46. % SPECIFIC LYSIS OF TARGETS EXPRESSING RRV VP7 PROTEIN BY RRV PRIMED C57BL/6 (H-2^b) EFFECTORS

Two 6 to 10 week old female mice C57BL/6 (H-2^b) were orally inoculated with 10^7 pfu rotavirus RRV (serotype 3) and 7 days later were sacrificed and the spleens removed. The splenocytes were *in vitro* stimulated to produce the effector cells as described in Figure 25.

Target cells (A) EL4 (H-2^b), (B) Ltk^- (H-2^k), (C) L-D^b (L-$2D^b$) or (D) L-K^b (H-$2K^b$) were loaded with ^{51}Cr, infected with WT vaccinia virus or recombinant vaccinia virus (vaccRRV.VP7) and the ^{51}Cr-release assays set up as described in Figure 25.

FIGURE45

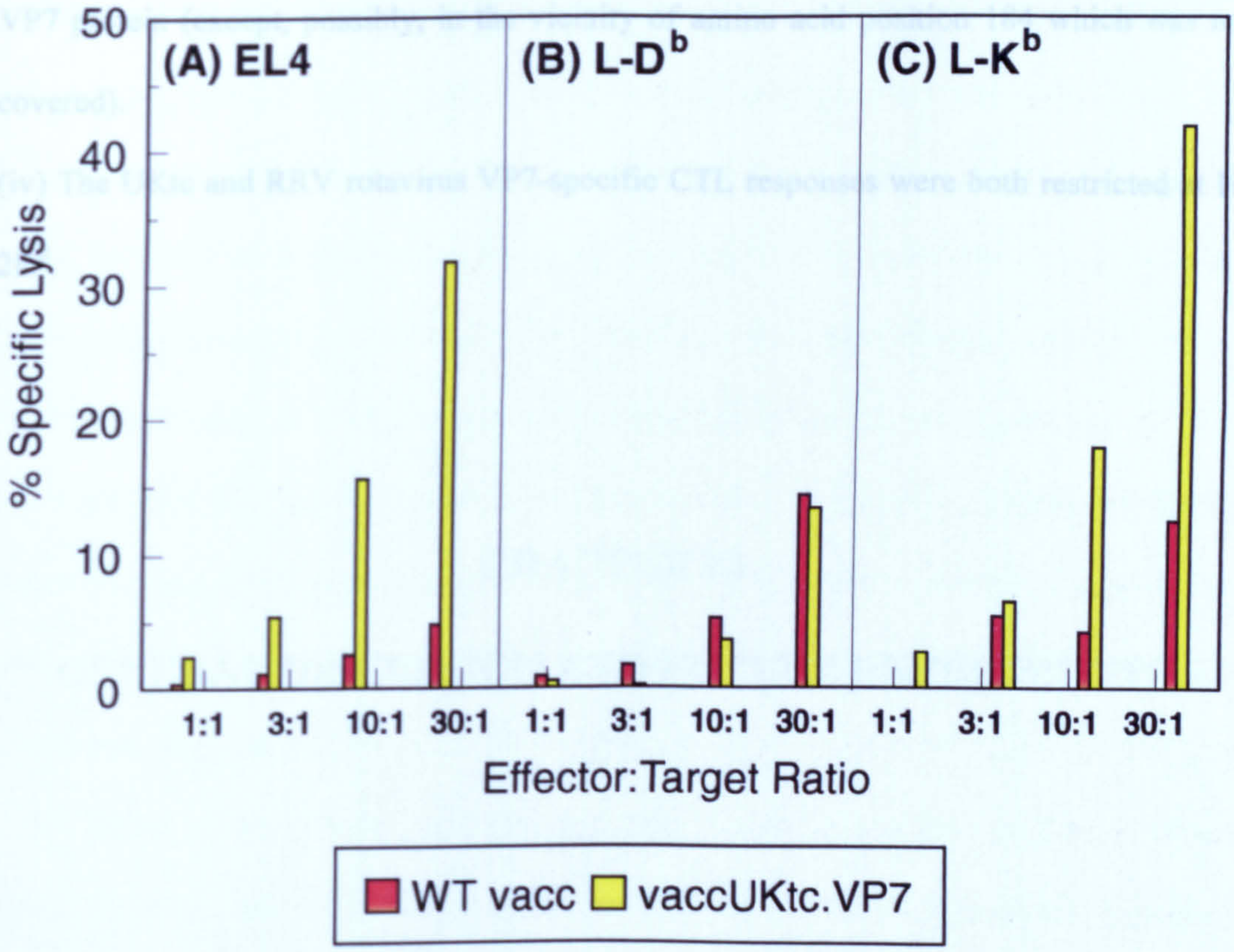
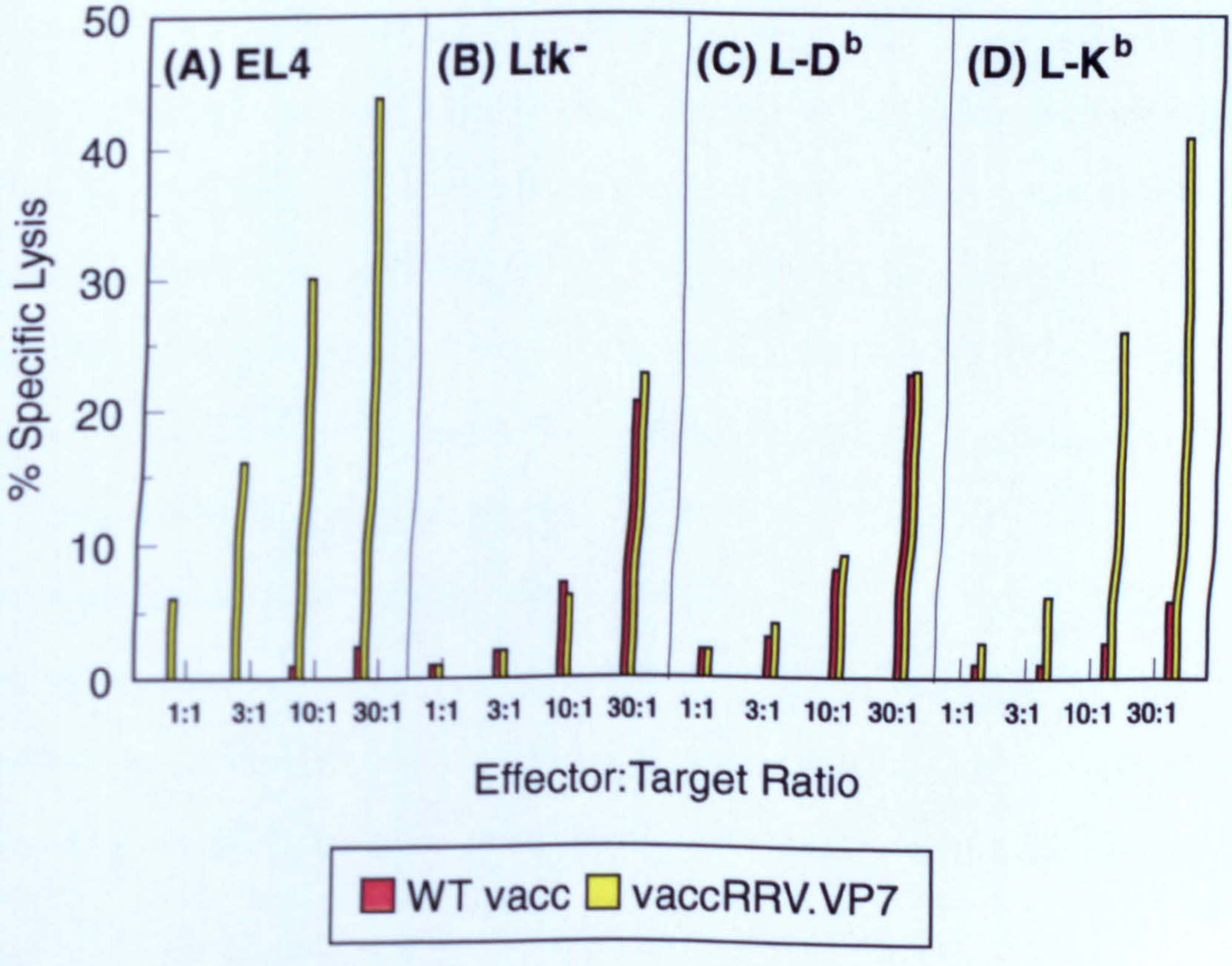


FIGURE 46



(iii) No major CTL epitopes were located between amino acids 117 and 232 of the UKtc VP7 protein (except, possibly, in the vicinity of amino acid position 184 which was not covered).

(iv) The UKtc and RRV rotavirus VP7-specific CTL responses were both restricted at H-2K^b.

CHAPTER 12

GENERAL DISCUSSION AND FUTURE EXPERIMENTS

Rotaviruses are the major etiological agents of severe infantile gastroenteritis, causing millions of cases of diarrhoea in children under five years of age and resulting in 850,000 deaths annually (WHO, 1989). Although rotavirus is rarely fatal in the developed world, nearly all children are infected by 3 years of age and rotaviral diarrhoea is believed to account for 209,000 hospitalisations annually in the United States (Ho *et al.*, 1988; Blacklow and Greenberg, 1991). A clean and plentiful water supply in developing countries would obviously help to prevent fatality by allowing for the replacement of the fluids and electrolytes lost during infection. However, even if this was provided, the scale of hospitalisations in developed countries, and the resulting economic burden (estimated to be 500 million to 1 billion dollars annually in the United States), make the development of a rotavirus vaccine a priority (Offit *et al.*, 1991a).

Initial studies focused on the rotavirus VP4 and VP7 proteins and established that both proteins could independently evoke a protective antibody response (Offit *et al.*, 1986b; Matsui *et al.*, 1989). Consequently, several vaccine trials using traditional types of vaccine (live, oral, attenuated viruses), or modified traditional vaccines (genetic reassortant viruses containing genes encoding VP7 and/or VP4 of selected human rotavirus strains in an animal rotavirus background), have been undertaken. As mentioned in chapter 3, the large field trials with these vaccines have revealed mixed efficacy, regardless of the development of neutralising antibodies (reviewed in Conner *et al.*, 1994). Nine VP7 (G) serotypes have been found to-date in humans, G1 to G4 being the most common (Beards *et al.*, 1992; Gerna *et al.*, 1992; Woods *et al.*, 1992), and several G serotypes can co-circulate within a population (Gomez *et al.*, 1990; Padilla-Noreiga *et al.*, 1990). Also, eleven VP4 (P) serotypes have been identified in group A viruses (Gorziglia *et al.*, 1990a) but, because P serotypes are difficult to differentiate serologically, VP4 is classified into nineteen genotypes using VP4 amino acid homology sequence analysis (Estes and Cohen, 1989). Nine VP4 genotypes have been identified in humans to-date and

genotypes 4, 8 to 10 and 14 are frequently associated with symptomatic infections (Qian and Green, 1991; Das *et al.*, 1993). Initial studies suggest that, like G serotypes, several VP4 genotypes can co-circulate within a population (Das *et al.*, 1994; Timenetsky *et al.*, 1994). From the total of VP7 serotypes and VP4 genotypes, and the capability of gene segment reassortment during mixed infection (VP4 and VP7 can independently segregate) (Offit *et al.*, 1986a), it is perhaps not surprising that the traditional vaccine strategies have provided mixed results.

CTLs appear to recognise serotype-independent epitopes in rotavirus proteins (Offit and Dudzik, 1988; Offit *et al.*, 1991a) which could be harnessed in vaccine design. However, although the antibody response to rotavirus has been studied extensively, little is known about the specificity and protection offered by the rotavirus-specific CTL response. The identification of antigenic epitopes capable of eliciting specific CTLs that can lyse infected cells is a fundamental step in the successful development of a CTL-promoting rotavirus vaccine, especially if those epitopes elicit a serotype/strain-independent CTL response.

Investigating the immune response to rotavirus in humans presents particular difficulties: (i) the prior rotavirus exposure history is unknown, (ii) the inoculation "take" rates depend on the maternal antibody status, (iii) the timing between inoculation and natural rotavirus exposure is variable, and (iv) variation occurs in the virulence of the natural rotavirus strains. Thus, initial studies are best performed in animals rather than humans and can, at the very least, provide insight for future studies in humans. In the present study, the mouse was used to examine the CTL response to rotavirus infection.

An animal model is required which mirrors the human immune response to rotavirus. Large animals, such as piglets and calves, accurately reflect human rotavirus illness (Bridger and Brown, 1981; Davidson *et al.*, 1983; Saif *et al.*, 1983), but are

expensive and difficult to maintain; caesarean delivery and gnotobiotic conditions often being required. Consequently, experiments requiring multiple animals are virtually impossible. Also, well-characterised immunological reagents for large animals may be expensive and difficult to obtain. Rabbits, although easier to handle than the large animals, are relatively expensive and also lack well-characterised immunological reagents (Conner *et al.*, 1991). The most important problem with all these animals is, however, the absence of inbred lines that are vital for the initial characterisation of the MHC class I restricted CTL response.

The mouse has many benefits over other animal models, the principal advantages being the availability of a number of inbred lines and a well-characterised immune system. Also, several commercial sources provide many high-quality reagents for detailed immunological studies. In addition, the mouse is priced modestly, requires less space and is easy to handle. The use of mice therefore allows a greater number of animals to be analysed per experiment leading to more meaningful data.

Although rotaviruses and influenza viruses have different structures and replication cycles, similarities do exist between the two viruses. Firstly, both rotavirus and influenza virus have segmented genomes which contain genes encoding two independently segregating surface proteins [in the case of influenza, hemagglutinin (HA) (neutralising) and neuraminidase (NA)] which are the basis for the classification into subtypes/serotypes (in the case of influenza, H1 to H14 and N1 to N9 subtypes with H1 to H3, and N1 and N2, identified in humans) (Murphy and Webster, 1990). Furthermore, the antibody response to both influenza virus and, probably, rotavirus generates a selection pressure directed to the surface proteins, which results in antigenic drift; the antibody response becoming progressively less effective as the virus changes antigenically (Kilbourne, 1973; Palombo *et al.*, 1993). Lastly, completely new subtypes/serotypes of both influenza and, probably,

rotavirus appear following interspecies transmission (Nakagomi and Nakagomi, 1989; Murphy and Webster, 1990; Das *et al.*, 1993).

Comparable to the recent rotavirus vaccine trials, the early influenza virus vaccines did not provide protection equal to natural influenza virus infection, even though virus-specific antibody levels were presumably boosted (Hoskins *et al.*, 1979). Focus then turned to the cell-mediated immunity and it was discovered that transferred CTLs could reduce influenza virus infection in the lungs of mice (Yap *et al.*, 1978). It was also found that the influenza virus-specific CTL response was subtype-independent (McMichael *et al.*, 1983b). The initial findings lead to a more detailed analysis of the CTL response to this virus with the aim of developing a CTL promoting vaccine. In humans, however, memory CTLs to influenza A virus decline within two to three years in the absence of further challenge (McMichael *et al.*, 1983a). The short lived nature of influenza virus-specific memory CTLs suggested that regular immunisations (perhaps annually) would be required to ensure a sufficiently high level of CTL protection against the challenge virus.

Although the lack of feasibility of a CTL-promoting vaccine to influenza virus has become apparent, the CTL response to this virus had been well defined and, with the above similarities to rotavirus, provided a relevant and well defined virus-specific CTL response to compare with rotavirus.

By contrast to influenza virus, the short lived nature of the CTL response to rotavirus should not be a problem as disease is restricted, in the main, to the first two years of life (Kapikian *et al.*, 1976). This apparent age restriction may be due to (i) the development of the gastric acid and pepsin secretions seen with increased age (Bass *et al.*, 1992), (ii) an age related change in the quantity of rotavirus binding receptors (Rienpenhoff-Talty *et al.*, 1982), or (iii) acquired immunity following asymptomatic infections of neonates with nursery rotavirus strains (Albert *et al.*, 1987). Therefore, in

principle, a CTL promoting vaccine could provide the host with protection during the two years of high disease susceptibility.

The lysis of target cells expressing individual rotavirus proteins by splenocytes from mice immunised with rotavirus has been described in this study. It might be argued that the cytolytic activity was due to other immune mechanisms since the splenocyte populations used in the ^{51}Cr-release assays contained many different cell types. However, previous studies using the same technique have showed that rotavirus-specific CTLs were indeed responsible for this activity because cytotoxic activity was (i) detected seven days after primary oral inoculation, (ii) not detected in uninoculated animals, (iii) specific for rotavirus infected target cells, (iv) eliminated by treatment with Thy 1.2-specific IgM and complement and (v) restricted by MHC class I antigens (Offit and Dudzik, 1988). Recently, magnetic beads were used to separate the splenocyte population from rotavirus UKtc inoculated C57BL/6 (H-2^b) mice, providing a positive population containing 78% CD8$^+$ cells and a negative population containing 0% CD8$^+$ cells. In ^{51}Cr-release assays, only the positive population lysed target cells expressing the UKtc NS53 or VP7 proteins, which directly showed that the cytolytic activity was mediated by CD8$^+$ CTLs (S. Stagg, personal communication).

In the present study, congenic mouse strains were used to detect if the CTL unresponsiveness to RRV VP7 in the Balb/c (H-2^d) and C3H/He,mg (H-2^k), but not C57BL/6 (H-2^b), mice strains was related to background differences other than the MHC class I haplotype, for example, the minor histocompatibility antigens. Studies in congenic C57BL/10 and Balb/c mice showed that the mouse strain restriction of the rotavirus-specific CTL response was dependent solely on the MHC class I haplotype of the mouse.

This study has shown that the virus strain origin of a particular protein affects whether it will be recognised by CTLs in a particular haplotype. For example, the UKtc NS53 was recognised in the H-2^b, but not in the H-2^d or H-2^k haplotype, whereas, RRV NS53 was recognised in both the H-2^b and H-2^d, but not the H-2^k haplotype. Indeed, Dharakul *et al.* (1991) did not detect a CTL response to RF NS53 using recombinant baculovirus expressed protein to inoculate H-2^d haplotype mice. This raises two points; firstly, to obtain a comprehensive picture, many different rotavirus strains would have to be examined in many different haplotypes to pin point the majority of CTL epitopes and, secondly, the proteins of different rotavirus strains that are specific for a CTL response can only be compared with caution and certainly not combined together.

It should be noted that previous studies on the CTL response to individual rotavirus proteins used different strains of rotavirus to inoculate mice than the present study [bovine RF (G6, VP4 genotype 1), Franco *et al.* (1994); simian SA11 (G3, VP4 genotype 1), Offit *et al.* (1994)], and so direct comparisons with these studies have to be viewed with caution.

For the above reason, the validity of the experiment which adoptively transfers into chronically infected SCID mice, CTLs generated from mice inoculated with baculovirus expressed individual rotavirus proteins, is doubted. In this experiment, VP1, VP2 and NS53 were from the RF strain, VP6 and NS28 were from the SA11 strain, VP4, VP7 and NS35 were from the simian RRV strain (G3, VP4 genotype 3) and the SCID mice were chronically infected with the murine EDIM strain (probably G3; VP4 genotype 16 or 17). The study found that protective CTLs were generated by baculovirus expressing VP1, VP4, VP6 and VP7 (Dharakul *et al.*, 1991). However, with so many different strains of rotavirus involved, the authors should have interpreted their results with more caution.

The present study was the first in which the CTL response to a full complement of rotavirus proteins from a single virus strain was examined. It has been known for some time that the virus protein recognised by CTLs depends on the MHC class I haplotype of the mouse (Bennink *et al.*, 1987; Whitton *et al.*, 1988b). For this reason, the immune response to the full complement of rotavirus proteins was examined in three different mouse MHC class I haplotypes: C57BL/6 (H-2^b), Balb/c (H-2^d) and C3H/He,mg (H-2^k).

In the H-2^b haplotype, the present study found a CTL response directed to the rotavirus UKtc VP7 and NS53 proteins. Most of the previous studies on CTL response to rotavirus have been performed in H-2^b haplotype mice and a VP7-specific CTL response has been detected often (Dharakul *et al.*, 1991; Offit *et al.*, 1991a; Franco *et al.*, 1994; Offit *et al.*, 1994). However, this was the first study (in collaboration with S. Stagg, personal communication) to detect a NS53-specific CTL response. Because of the qualitative nature of the ^{51}Cr-release assay, the relative levels to which rotavirus-specific CTLs recognise UKtc VP7 compared to UKtc NS53 in the H-2^b haplotype remains to be determined.

The CTL response previously detected to UKtc VP4 in the H-2^b haplotype (Offit *et al.*, 1991a) was not detected in later studies (Offit *et al.*, 1994) or indeed in recent experiments (S. Stagg, personal communication). Also, the CTL response previously found to the VP6 protein, of both the SA11 and RF strains (Offit *et al.*, 1991a; Franco *et al.*, 1994), was not detected using UKtc virus. Furthermore, the CTL responses to RF VP2 and VP3, using baculovirus expressed proteins to inoculate mice, were not detected using corresponding UKtc proteins.

In the case of the inoculation of mice with recombinant baculovirus expressed individual rotavirus proteins, it appears that the available CTL repertoire is greater than that induced by infectious rotavirus itself. This may arise from the difference in the presentation to the immune system of endogenously synthesised proteins compared to

exogenous proteins. However, unlike these previous studies, this study has detected the rotavirus proteins that elicit a CTL response following whole active rotavirus inoculation of mice and is, therefore, more indicative of the actual CTL response that follows a natural rotavirus infection. Therefore, in the design of a CTL-promoting rotavirus vaccine, the results of this study are probably of more value than the results gained using recombinant baculovirus expressed proteins or recombinant vaccinia virus to inoculate mice.

In the H-2^d mouse haplotype, the current study detected only a UKtc VP3-specific CTL response. Interestingly, Franco *et al.* (1994) were unable to detect a CTL response using the baculovirus expressed VP3 (or VP1, VP2 or VP6) to inoculate H-2^d mice, perhaps, because the RF strain was examined in this case. In variance to Franco *et al.* (1994), but using the same methods, Dharakul *et al.*, (1991) did find a RF VP1-specific CTL response in this haplotype. Dharakul *et al.* (1991) also found a CTL response to VP6, but of the SA11 strain in this case.

In addition, Dharakul *et al.* (1991) found RRV VP4 and VP7-specific CTL responses in the H-2^d mouse haplotype. Of note, the present study failed to detect a RRV VP7-specific CTL response in this haplotype and, as described above, this may be due to the greater CTL repertoire induced by recombinant baculovirus protein inoculation.

In the H-2^k mouse haplotype, the current study also detected only a UKtc VP3-specific CTL response. This agrees with preliminary results cited by Franco *et al.* (1994) in which a rotavirus-specific CTL response was generated using the baculovirus expressed RF VP3 (but not VP1, VP2 or VP6) to inoculate H-2^k mice.

To summarise, examination of the full complement of rotavirus UKtc proteins revealed a NS53 and VP7-specific CTL response in the H-2^b mouse haplotype and a VP3-specific CTL response in the H-2^d and H-2^k mouse haplotypes. Unlike the present work, studies concerning the CTL response to influenza virus have not covered the full complement of proteins from one subtype. Using various methods to express individual

influenza virus proteins (of many different subtypes) in different mouse haplotypes, virus-specific CTLs have been generated against:- the surface glycoproteins, HA (Bennink *et al.*, 1984) and NA (Wysocka and Hackett, 1990), the internal proteins, matrix protein (Reay *et al.*, 1989), nucleoprotein (NP) (Townsend *et al.*, 1984; Yewdell *et al.*, 1985), and the three viral polymerases (PA, PB1 and PB2) and the non-structural protein (NS1) (Bennink *et al.*, 1987). In common with rotavirus, the CTL recognition of individual influenza viral epitopes depended on the mouse haplotype (Bennink *et al.*, 1987; Reay *et al.*, 1989), a situation also found with lymphocytic choriomeningitis virus (LCMV) (Whitton *et al.*, 1988b).

Resulting from detection of a rotavirus UKtc VP3-specific CTL response in the H-2^d and H-2^k (but not H-2^b) haplotypes, the CTL response to VP3 was examined in more detail. Since peptides which bind to MHC class I molecules have a unique motif, it appears that UKtc VP3 contains at least two CTL epitopes (presented by H-2^d and H-2^k). Franco *et al.* (1994) also found a VP3-specific CTL response in two haplotypes when using recombinant baculovirus expressed protein to inoculate mice and this suggested that RF VP3 also contained at least two epitopes (presented by H-2^b and H-2^k in their case).

The location of both the RF VP3 and UKtc VP3 epitopes expressed by the H-2^k haplotype is not known and further studies may find that the CTL response to these two proteins is strain-independent. However, having established a CTL response to UKtc VP3 in both H-2^d and H-2^k mouse haplotypes, this study suggested that the VP3 response was strain-dependent, at least between RRV and UKtc, regardless of the haplotype (H-2^d or H-2^k). Although it is not known if a CTL response is directed to RRV VP3, this result suggests that the epitope(s) in VP3 are not conserved between rotavirus strains (i.e., in variable region(s) of the protein). Of note, Franco *et al.* (1994) used allele-specific motifs

to predict the H-2^b epitope(s) of the RF VP3 and found a response to a peptide mapping to amino acids 585 to 593 which was restricted at K^b.

In common with the responses to VP3 of rotavirus, CTLs are induced to the internal, conserved non-glycosylated proteins of many viruses including; influenza (Townsend *et al.*, 1984; Yewdell *et al.*, 1985), respiratory syncitial virus (RSV) (Bangham *et al.*, 1986) and vesicular stomatitis virus (VSV) (Puddington *et al.*, 1986; Yewdell *et al.*, 1986). Indeed, the responses to the conserved internal proteins often predominate during the polyclonal CTL response and are frequently strain-independent. It is unusual that, at least between the RRV and UKtc rotavirus strains, the VP3-specific CTL response does not appear to be cross-reactive.

Following the detection of a rotavirus UKtc NS53-specific CTL response in the H-2^b haplotype and a RRV NS53-specific CTL response in the H-2^b and H-2^d haplotypes, the CTL response to NS53 was examined in more detail. Because the peptides which bind to MHC class I molecules have a unique motif, it appears UKtc NS53 contains at least one CTL epitope (presented by H-2^b), whereas, RRV NS53 contains at least two CTL epitopes (presented by H-2^b and H-2^d).

This study found that the NS53-specific CTL response was strain-dependent (between UKtc, RRV and Hochi), regardless of the inoculating strain (UKtc or RRV) and irrespective of the haplotype (H-2^b or H-2^d). Although it is unknown if a CTL response is directed to Hochi NS53 in either haplotype, this result suggests that the epitope(s) on NS53 are not conserved between rotavirus strains (i.e., in variable regions of the protein).

The study also showed that the UKtc NS53-specific CTL response was restricted at H-2D^b. The RRV NS53-specific CTL response in the H-2^b haplotype could not be clearly defined as D^b or K^b restricted and this suggests that at least two CTL epitopes are presented, at least one H-2D^b and H-2K^b, respectively. This hypothesis is supported by the

earlier suggestion that RRV NS53 contains at least two CTL epitopes due to the CTL response in two different haplotypes. In comparison, the simian virus 40 large tumour (T) antigen contains three H-2D^b and one H-2K^b restricted CTL epitopes (Mylin *et al.*, 1995).

Not only was the current study the first to show a H-2D^b restricted, strain-dependent CTL response directed at the UKtc NS53 protein, but, at least one CTL epitope has been located to the first 150 amino acids of this protein.

The strain-dependent CTL response to rotavirus NS53 is in contrast to the strain-independent CTL response of the non-structural proteins of both influenza virus NS1 (Kuwano *et al.*, 1990) and hepatitis C virus (HCV) NS5 (Shirai *et al.*, 1992). However, it should be noted that the influenza virus NS1 and HCV NS5 proteins are both relatively conserved between isolate groups, whereas rotavirus NS53 protein has great genetic diversity between strains (for example, UKtc and RRV NS53 have only 37.8% amino acid conservation) (Xu *et al.*, 1994) and, thus, the absence of strain-independent CTL epitopes is not surprising. It is possible that the genetic diversity of NS53 is a result of selection pressure bestowed by the cell-mediated immune response, as has been shown to be the case in Epstein Barr virus (EBV), LCMV and HIV-1 (Pircher *et al.*, 1990; Campos-Lima *et al.*, 1993; Couillin *et al.*, 1994). It is believed that induced mutational changes either prevent the binding of the viral CTL epitopes to the MHC molecules, or prevent the T-cell receptor (TCR) from binding to the MHC-peptide complex. In either event, a sub-population of CTLs no longer recognise the virally infected cells and, depending on the hierachal control of other CTL epitopes, the virus replication may continue undetected (Lewicki *et al.*, 1995; Moskophidis and Zinkernagel, 1995).

The present study found that the UKtc and RRV VP7-specific CTL response was restricted to the H-2^b mouse haplotype. The VP7-specific CTL response in this haplotype has been well documented using the UKtc, RRV, SA11 and RF rotavirus strains (Offit *et al.*, 1991a;

Franco *et al.*, 1993; Offit *et al.*, 1994). An RRV VP7-specific CTL response was also found in the H-2^d haplotype when baculovirus expressed protein was used to inoculate mice (Dharakul *et al.*, 1991).

A previous study had shown that the VP7-specific CTL response was serotype-independent between serotypes 3 (SA11 and RRV) and 6 (UKtc) (Offit *et al.*, 1994). However, because fourteen different VP7 serotypes have been classified to-date, it was decided to extend the previous findings to cover a large number (7) of the serotypes. This study found that the VP7-specific CTL response was serotype-independent between all the rotavirus serotypes tested, regardless of the immunising serotype (3 or 6) in the H-2^b haplotype. Since the VP7-specific CTL response is serotype-independent between seven of the fourteen serotypes, it would appear reasonable to hypothesise that the CTL response probably cross-reacts between all fourteen VP7 serotypes, regardless of the inoculating rotavirus serotype. These results suggest that the CTL epitope(s) on the VP7 protein are conserved between rotavirus strains.

The study also showed that the serotype-independent UKtc and RRV VP7-specific CTL epitopes were both restricted at H-2K^b. This disagrees with Offit *et al.* (1994) who found that either the UKtc or SA11 (they do not specify) VP7-specific CTL response was restricted at H-2D^b and, interestingly, that RRV-specific CTL recognition of RRV infected target cells was restricted at H-2D^b (Offit and Dudzik, 1988). By contrast, and in agreement with the results reported here, the immunodominant epitope on RF VP7 (as well as VP3 and VP6) was found to be restricted at H-2K^b (Franco *et al.*, 1993/1994). Therefore, the conclusions reached by Offit and colleagues have to be questioned.

Using allele-specific motifs to predict MHC class I H-2^b epitopes, a synthesised peptide covering amino acids 33 to 40 of the rotavirus RF VP7 protein (which overlaps the H2 signal peptide located between amino acid positions 33 to 48) was recognised by rotavirus-specific CTLs (Franco *et al.*, 1993). This epitope was found to be restricted at H-

$2K^b$ and, in addition, the region of the VP7 protein containing this epitope is conserved, although not completely conserved, between different rotavirus strains (see Table 10). In the light of this, the view emerged that this region defined the VP7 serotype-independent CTL epitope, however, no experimental evidence for this had been reported. The present study showed that UKtc VP7 contains a serotype-dependent CTL epitope between amino acids 13 and 48; this region containing the immunodominant epitope detected previously which is fully conserved between the UKtc and RF rotavirus strains (see Table 10). This suggests that the immunodominant CTL epitope found by Franco *et al.* (1993) was not the serotype-independent epitope.

Table 10. Comparison of the VP7 amino acid sequence (between amino acid positions 33 and 40) corresponding to the H-$2K^b$ restricted CTL epitope detected by Franco *et al.* (1993). Amino acid residues which differ from the rotavirus RF strain sequence are shown in bold (Estes and Cohen, 1989).

Rotavirus strain	VP7 serotype	VP7 sequence between amino acid positions 33 to 40
RF	6	I I Y R F L L I
Wa	1	I I Y R F L L I
RRV	3	I I Y R F L **F** I
ST3	4	I I Y R **I A F V**
OSU	5	I I Y R F L L **V**
UKtc	6	I I Y R F L L I
69M	8	I **L** Y **H** F L L **F**
WI-61	9	I I Y R F L L **L**
A64	10	I I Y **K** F L L I

These results have shown that, although CTL epitopes can be predicted from the amino acid sequence of a protein (Franco *et al.*, 1993), a better method is to examine progressively smaller protein fragments (as utilised in this study). The problem of predicting CTL epitopes is highlighted by the fact that HCV epitopes exist in which no binding motif has yet been proposed (Koziel *et al.*, 1995). In addition, apart from the three immunodominant LCMV H-2^b restricted CTL epitopes, only three out of a further thirty-four predicted CTL epitopes were functional and even those were not recognised *in vivo* following the mutation of the immunodominant CTL epitopes (Oldstone *et al.*, 1995).

In summary, VP7 contains at least one serotype-dependent and one serotype-independent epitope recognised by CTLs and all the epitopes are restricted at H-$2K^b$. The rotavirus VP7-specific CTL response is in contrast to viruses such as influenza (Townsend *et al.*, 1984), RSV (Bangham *et al.*, 1986) and VSV (Puddington *et al.*, 1986; Yewdell *et al.*, 1986) where the surface glycoprotein-specific CTL response is either a minor component of the polyclonal CTL response or undetectable. It should be noted that although the majority of the CTL response to the influenza virus HA is subtype specific (Townsend *et al.*, 1984), minor subtype-independent CTL epitopes have also been detected in this protein (Saikh *et al.*, 1995). Conversely, the serotype-independent LCMV envelope glycoprotein-specific CTL response mirrors the rotavirus VP7-specific CTL response (Whitton *et al.*, 1988a/b).

The identification of those rotavirus protein(s) involved in stimulating a CTL response may be important in the development of vaccines based on vectors expressing individual rotavirus genes, particularly if the proteins concerned evoke serotype-independent CTLs. Mice inoculated with an infectious virus or with recombinant vaccinia virus expressing VP7 induce VP7-specific serotype-independent CTLs (Offit *et al.*, 1994) and, with the

development of naturally attenuated poxvirus vectors for use in humans, these types of expression systems may be utilised in future vaccine design (Taylor *et al.*, 1992). With regards to the influenza virus nucleoprotein (NP) (which contains the immunodominant subtype-independent CTL epitope), there is still debate as to the level of protection offered by immunisation with vaccinia virus recombinants containing this protein. Immunisation with recombinant vaccinia virus expressing NP offered protection to mice from heterotypic virus challenge (Endo *et al.*, 1991), however, this protection has not been found by other investigators (Lawson *et al.*, 1994). Immunisations with recombinant vaccinia viruses containing proteins which elicit virus-specific CTLs have also been found to elicit protection against subsequent virus infections of both LCMV and RSV (Hany *et al.*, 1989; Kulkarni *et al.*, 1993).

In contrast to antibody producing B-lymphocytes, CTLs do not require conformationally correct protein presentation for their stimulation and, indeed, a subunit vaccine could potentially contain individual CTL epitopes rather than complete proteins. Thus, utilising CTL epitopes might bypass the difficulty of achieving correct protein conformation found in heterologous expression systems. Since the rotavirus protein used as a source of CTL determinants is dependent on the haplotype of the mouse, an effective subunit vaccine for use in an outbred population would probably have to contain a variety of CTL epitopes. Indeed, coupling of LCMV CTL-specific epitopes within vaccinia virus to form a "string-of-beads" vaccine was found to not only confer protective CTL responses in several MHC backgrounds but, also, to enhance the CTL response within individual MHC backgrounds (Whitton *et al.*, 1993). In addition, the efficient use of individual rotavirus-specific CTL epitopes in a subunit vaccine requires coupling to Th-lymphocyte epitopes. However, the search for immunodominant rotavirus Th-lymphocyte epitopes is not necessary because coupling to defined Th-lymphocyte epitopes of an unrelated virus (for example, HIV) is sufficient to provide the additional help (Shirai *et al.*, 1996). Shirai

and colleagues also speculated that the attachment of these epitopes may broaden the range of MHC haplotypes in which the potential vaccine would be effective.

A recent discovery was that virus-specific CTLs could be generated in animals by intravenous, intra-muscular and gene gun immunisation of DNA vectors (Yokoyama *et al.*, 1995; Yasutomi *et al.*, 1996). For example, heterotypic protection of mice from influenza virus infection was provided by the intra-muscular immunisation of a DNA plasmid vector encoding the influenza NP protein (Ulmer *et al.*, 1993). In addition, the intra-muscular immunisation of a DNA plasmid containing the HBV small surface antigen (HBsAg), into a mouse haplotype (H-2^b) previously considered a non-responder to the HBsAg, revealed a CTL response to this protein (Schirmbeck *et al.*, 1995). The conclusion of this work was that, depending on the method of antigen delivery, CTL subunit vaccines have the potential to elicit heterotypic protection and overcome the non-responsiveness of particular proteins in certain haplotypes.

Future experiments leading directly on from this work would include precisely locating the serotype-independent epitope(s) on VP7 and the strain-dependent CTL epitope(s) on NS53, for each appropriate mouse haplotype. Also the location of the VP3-specific CTL epitopes should be determined and the strain-specificity of these epitopes confirmed. Furthermore, the MHC class I locus of restriction with regard to rotavirus proteins in mouse haplotypes other than H-2^b have yet to be determined. It would also be interesting to see if immunisation with the recombinant vaccinia viruses expressing VP3, NS53 and VP7 could protect infant mice from subsequent rotavirus disease. Different vaccination strategies could be examined with the recombinant viruses given either singularly (in the appropriate mouse haplotype) or in a combination to perhaps elicit a mouse haplotype-independent protective CTL response.

Despite the above details that remain unresolved, the information to be gained on the CTL response to rotavirus using the inbred mouse model has virtually been completed. The CTL response appears complicated and varies according to both the mouse haplotype and rotavirus strain. The next step is to look at the CTL response to rotavirus in an outbred population. This could also be done in mice, however, because a rotavirus vaccine is urgently required for humans, it would be more practical to look at the CTL response to all the rotavirus proteins using humans as the outbred population. Indeed, the ^{51}Cr-release assay for measuring CTL activity to individual virus proteins using targets infected with vaccinia virus recombinants, has been performed using human CTLs (Tamaki *et al.*, 1995). Autologous EBV-transformed B-lymphoblastoid cell lines from each patient are normally established by *in vitro* transformation of the peripheral blood B-lymphocytes with the supernatant from an EBV-producing marmoset cell line. The autologous B-lymphocyte blasts are used as both the antigen presenting cell during the *in vitro* stimulation of CTLs and as the target cells in the ^{51}Cr-release assays.

The rotavirus-specific CTL response in humans has not been examined to-date and this probably stems from the fact that adult volunteers have an unknown immunological history to rotavirus. Since memory CTLs are believed to decline within three or four years, a CTL response may not be detected in adult volunteers. One may argue that the probable asymptomatic rotavirus re-infections throughout the life of an individual may help to preserve rotavirus-specific memory CTLs, however, the development of an antibody response may prevent sufficient infection for stimulation of memory CTLs. Consequently, immunisation of adult volunteers with rotavirus prior to the examination of their CTLs may not be sufficient in generating a rotavirus-specific response. It would therefore seem more prudent to perform the initial experiments on the rotavirus-specific CTL response in humans, although technically more difficult, using infants following their primary rotavirus infection.

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